GENETIC DISSECTION OF SEX DIFFERENCES IN HUMAN BRAIN AND BEHAVIOUR

Geoffrey Tan

Wellcome Trust Centre for Neuroimaging

Department of Molecular Neuroscience

Institute of Neurology

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Prof Richard SJ Frackowiak, Prof Nicholas W Wood and Dr John Ashburner

Declaration

I, Geoffrey Tan, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

The importance of sex differences in the brain and behaviour is indisputable. It forms the basis for differences in risk across a range of neurological and psychiatric disorders, as well as gender roles within society. The classical approach to investigating sex differences primarily involves comparisons between males and females. While informative for characterizing the wide array of sexually dimorphic traits, straight comparisons are insufficient to elucidate specific molecular contributions due to the multiplicity of confounding factors. Discrete genetic polymorphisms can be used to investigate variance in these traits due to sex-related molecular factors independent of confounds of sex.

This thesis applies candidate genetics to understand the specific contributions of molecular components of the sex hormone pathways to sexual dimorphism in brain structure, personality and cognition. A cohort of 384 individuals were recruited to undergo MRI brain scans, cognitive and personality testing. They also provided blood samples for candidate genotyping in polymorphisms in genes for the androgen receptor, oestrogen receptors, progesterone receptor and aromatase enzyme that converts testosterone to oestrogen. Voxel-based morphometry was used to characterise regional differences in brain volume accounted for by these polymorphisms and the relationship to sex differences in brain volume. Diffusion tensor imaging was then used to determine variation in white matter integrity and structural connectivity due to these polymorphisms. Sex differences in personality and cognition are further investigated in terms of correlations with the polymorphisms and brain structure. Finally an endophenotype approach was used to investigate differential risk for conditions such as Alzheimer's disease and depression between sexes through related brain and personality-based traits. The neural and molecular genetic mechanisms underlying this risk are inferred from correlations with brain-based measures and genotype. The strengths and weaknesses of this approach and the scientific implications of this work to gender research are discussed.

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CONTENTS

FIGURES

TABLES

GLOSSARY

Brain regions

GM: Grey matter

WM: White matter

CSF: Cerebral spinal fluid

PFC: Prefrontal cortex

BA: Brodmann area

TL: Temporal lobes

ACC: anterior cingulate cortex

AI: anterior insula

IFG: inferior frontal gyrus

OFC: orbitofrontal cortex

PFC: prefrontal cortex

DLPFC: dorsolateral PFC

DMPFC: dorsomedial PFC

MPFC: medial prefrontal cortex

VLPFC: ventrolateral PFC

VMPFC: ventromedial PFC

SS: Somatosensory

SLF: Superior longitudinal fasciculus

BA10: Rostral prefrontal cortex

IFOF: Inferior fronto-occipital fasciculous

IFG: Inferior frontal gyrus

BA21: Lateral temporal cortex

BA38: Temporo-polar area

BA17/BA18: Occipital cortex

TP: temporal pole

TPJ: temporoparietal junction

Methodology

BOLD: blood oxygen level dependent

DTI: diffusion tensor imaging

FA: fractional anisotropy

FWE: family-wise error

PET: Positron emission tomography

MRI: Magnetic resonance imaging

3D-MDEFT: Three dimensional Modified Driven Equilibrium Fourier Transform

SVC: Small volume correction

GLM: General linear model

ROI: Region of interest

FLIRT: FMRIB Linear Image Registration Toolbox

FDT: FMRIB Diffusion Toolbox

TBSS: Tract-based spatial statistics

MNI: Montreal Neurological Institute space

SPM: Statistical parametric maps

Genetics techniques

DNA: deoxyribonucleic acid

RNA: ribonucleic acid

bp: Base pairs

SNP: Single nucleotide polymorphism

PCR: Polymerase Chain Reaction

RFLP: Restriction fragment length polymorphism

Genes, polymorphisms and molecular biology

MAOA: Gene for monoamine oxidase A

MAOA VNTR:

PR: Gene for the progesterone receptor

PROGINS: Minor allele for alu insertion polymorphism within PR

ER: Oestrogen receptor

ERE: Oestrogen Responsive Elements

ESR1: Gene for the oestrogen receptor alpha

ESR1 TA repeat, ESR1(TA)n: TA repeat polymorphism of ESR1

ESR2: Gene for the oestrogen receptor beta

ESR2 CA repeat, ESR2(CA)n: CA repeat polymorphism of ESR2

AR: Gene for the androgen receptor

AR CAG repeat, AR(CAG)n: CAG repeat polymorphism in exon 1 of AR

CNTNAP2: Gene for Contactin (CNTN)-associated protein-2

XIST: X Inactivation-Specific Transcript

RNA: Ribonucleic acid

SRY: Sex-determining region Y

Sry: Mouse homologue of SRY

GnRH: Gonadotrophin Releasing Hormone

CYP19, CYP19A1: Gene for aromatase or Cytochrome P450, Subfamily XIX, CYP19

CYP19(TTTA)n: TTTA repeat polymorphism of CYP19

PR, PGR: Progesterone receptor

BMD: Bone mineral density

2D:4D: Second digit to fourth digit ratio

5-HT: serotonin

5-HTTLPR: serotonin transporter-linked promoter region

SS/LL: Short short or Long long alleles

ASPM: Abnormal Spindle-like, Microcephaly-Associated

Diseases

AD: Alzheimer's Disease

ASD: Autistic spectrum disorder

PDD-NOS: Pervasive developmental disorder- Not otherwise specified.

Personality

BSRI: Bem sex role inventory

NEO-PI-R: NEO Five-factor inventory revised

STAXI: Spielberger State-Trait Anger Expression Inventory

KSP: Karolinska Scales of Personality

TPQ: Tridimensional Personality Questionnaire

STAI: Spielberger State-Trait Anxiety Inventory

BEQ: Berkeley Expressivity Questionnaire

CHAPTER 1

INTRODUCTION

1.1 Context

Sex underlies the greatest and most consistently observed individual differences in human brain and behaviour. Differences between males and females have been observed from such basic traits as brain volume to higher components of social interaction such as empathy. It remains unclear to what extent these differences arise from societal roles arising from gender identity and to what extent they may be explained by specific molecular and neural factors that may have been important in conferring a survival advantage in the evolution of mankind.

1.1.1 Premise

The biological components defining sex vary across the population even within-sex and this variation is known to be mediated in part by specific gene variants. Thus by inference, such variants should similarly modulate stable sexually dimorphic traits, albeit to a smaller extent.

1.1.2 Aim

This thesis attempts to identify specific molecular factors that may underlie various facets of sexual dimorphism by investigating genetic variation in traits and endophenotypes for disease known to manifest a difference between males and females.

1.2 Background

1.2.1 Evolutionary basis for sexual dimorphism

Sexual reproduction has been a major driving force in evolution and accounts for the genetic component of within-species variability that has allowed adaptation of higher organisms to changing conditions. To attain this advantage, organisms have developed dimorphic individuals within species to allow the mixing and matching of genetic material during reproduction. Indeed substantial evidence suggests that in humans, evolution is largely male-driven (Makova and Li, 2002). Thus the broad similarity of non-sexual differences across species characterising the male and female members thereof may be more than a coincidence. Proponents of theories of sexual selection claim teleologically that rather than being a random set of characteristics, sexual dimorphism has evolved to maximise reproductive capacity by shaping particular roles that define mate selection, rearing of offspring and subsistence (Darwin, Jones and Ratterman, 2009, Shuster, 2009, Buss, 1989, Geary, 1998). This in turn could potentially form the basis for several types of social behaviour and cooperative relationships within the animal kingdom. While clearly aspects of these roles have changed in modern human society, one may draw a possible connection between human sexual dimorphism and some of these traditional roles within the animal kingdom. By identifying the particular factors that specify these roles, researchers may start to unravel the basis for the evolution of these differences and understand how manipulation of these factors can change these fundamental facets of animal behaviour.

1.2.2 Relevance to gender roles in society

In this thesis, I adopt the definitions of sex and gender used by the World Health Organisation (2010):

"Sex" refers to the biological and physiological characteristics that define men and women.

"Gender" refers to the socially constructed roles, behaviours, activities, and attributes that a given society considers appropriate for men and women.

So what relevance might this hold for humans in modern society? A large survey of gender research in the past century found that sex differences exist in behaviour within relationships, domestic roles, education and the workforce. Many or all of these may have environmental or cultural influences, and it remains to be seen how significant the biological or genetic contribution is in these domains. As mentioned, biologists often attempt to explain these differences in a framework of 'differential reproductive strategies' between sexes where there is male competition with males attempting to mate as often as possible and female choice, with females wanting to mate with the 'highest quality' male in order to optimise survival of their progeny (Buss, 1989, Geary, 1998). It is believed that this is a result of increased reproductive cost for females who must undergo gestation and often are primarily responsible for the subsequent well-being of their offspring. In support of this, human males report both stronger sex drive and desire for numerous sex partners as compared to females. Clear differences exist in the characteristics that each sex reports finding attractive in a mate as well-males prefer female mates younger than themselves and vice versa; males prefer mates who are physically more attractive; and females prefer mates who are taller, have higher social status and who have access to more resources. Domestically, females report providing more care to offspring, others in general and are more likely to have intimate friendships. In education, females spend more time studying and appear to achieve better grades generally, while males are more likely to major in engineering or the physical sciences in the undergraduate years. At work, studies support sex-typical stereotypes of occupational preferences with more males in law enforcement,

politics, science, engineering and professorships and more females in nursing (Maccoby and Jacklin, 1974, Ellis, 2008).

1.2.3 Clinical relevance

Implications of finding specific biological factors in sexual dimorphisms are more direct within the field of clinical medicine. As in a range of general medical conditions, sex differences in risk are common across psychiatry and neurology and identification of specific factors mediating such risk can aid in early assessment and development of interventions. Here I discuss a few of the traits relevant to the context of the thesis. In psychiatry, males broadly have more behavioural disturbances and appear to display more externalising behaviour, while females ruminate more over negative emotional experiences that relates to higher risk for general depression post-pubertally. While females experience more psychological distress and mental problems in general and more commonly have eating disorders and anxiety-related conditions such as phobias and panic disorder, males have higher rates of psychotic episodes, autistic spectrum disorders, attention deficit hyperactivity, antisocial personality disorders and alcoholism. Many of these conditions have a range of related traits in normal individuals that can be considered to be endophenotypes, either mediating risk or modulating the disease (Gaub and Carlson, 1997, Ellis, 2008, Aleman et al., 2003, Grilo et al., 1996, Piccinelli and Wilkinson, 2000).

In neurology, females probably have higher rates with earlier onset of Alzheimer's disease and general dementia, while males probably have higher rates of epilepsy, Parkinson's disease and prosopagnosia, the inability to recognise faces and their particular characteristics over other objects (Everhart et al., 2001, Haaxma et al., 2007, Launer et al., 1999). Females also appear to have more headaches generally, particularly for migraine headaches with only cluster headaches more common in males. Females also appear to experience greater pain than males even after controlling for gynaecologic and reproductive factors (Ellis, 2008). Identification of biological factors underlying differential risk between sexes can clarify mechanisms underlying these often complex and poorly

understood conditions and may pave the way for interventions that can either decrease risk or modify the course of disease. Many of these factors are directly influenced by frequently prescribed hormonal interventions such as oral contraception and hormone replacement therapy. Thus there is utility in investigating the proximate basis for observed sex differences in disease risk, which is the second approach in this thesis. Specifically, this thesis investigates neural and behavioural variation across individuals with genetic polymorphisms found to confer risk for disease in a sex-specific manner or where the disease involved has a significant disparity in prevalence between sexes.

1.2.4 The complexity of the gene-environment interaction

Many of these observed differences may not necessarily have a genetic or biological basis and are likely to be shaped by cultural expectations or opportunities and environmental influences. Indeed there is no doubt that the media propagates some of these stereotypes with males more likely to be portrayed in traditionally masculine roles, working outside of home and as being physically violent, while females are more likely to be portrayed as working inside the home (Ellis, 2008). Here the investigator encounters a chicken and egg paradox, where it is unclear whether such cultural influences and stereotypes are caricatures of true, albeit somewhat more subtle, differences or whether those roles came about arbitrarily and engendered differences that I now observe. The dissection of this gene-environment interaction, where oftentimes complex epistatic influences mean that genetic effects can underlie vulnerability to certain environmental factors and environment modulates the expression of genetic effects, is therefore by no means trivial. While heritability studies can help to understand the degree to which these traits are shaped by genetic or 'innate' components, such estimates are shaped by the degree of environmental variability itself. Beyond the identification of candidate phenotypes for genetic or biological investigation, heritability estimates cannot directly inform mechanistic inferences on these differences. With a substantial emphasis on gender equality in modern society, understanding the biological underpinnings of these differences rather than favouring bigotism and misogyny or misandrogyny can potentially provide factual information useful to determining ways that such equality can be realistically achieved

while acknowledging that males and females are not the same. Nonetheless, it is beyond the scope of this thesis to explore many these sociological implications in depth or to make any argument for a particular value judgement in the interpretation of any findings (Geary et al., 2000). Instead the following questions are posed: Is there an association between specific components of the molecular pathways underlying sex differences, particularly in the sex hormone receptors and these traits which biologists argue have arisen through evolution and therefore have a genetic basis? What are the neural mechanisms through which these molecular factors bring about the observed differences in behaviour?

So what then are the factors underlying sex differences and how do they relate to brain and behaviour? I start by discussing some of the biological factors underlying sexual dimorphism in order to consider potential candidates before going further to discuss my general approach.

1.2.4.1 Biological factors

Steroid hormones have multiple roles throughout the normal lifespan, from the determination of sex in early embryonic development to puberty, and influences on various facets of ageing. In addition to hormones however, sex differences can be caused by sexually dimorphic gene expression, Y-chromosome specific effects and X-linked gene dosage, as well as by environmental and social influences (Davies and Wilkinson, 2006).

1.2.4.2 Sex chromosomes

Fundamentally, the plethora of differences between males and females originates from the make-up of the sex chromosomes. In mammals, specifically eutherians or placental mammals, males possesses an X-chromosome and a Y-chromosome; females, two X-chromosomes. Some genes, found in pseudoautosomal regions of the sex chromosomes, are present in both X and Y chromosomes and thus recombine during meiosis (Goodfellow

and Lovell-Badge, 1993). Others are either present only on the Y-chromosome and are thus male-specific, or present on the non-pseudosomal regions of the X-chromosome with differences in gene-dosage between males and females (Goodfellow and Darling, 1988, Wilson and Makova, 2009). In the latter, differences in expression or effective gene-dosage rely upon a process known as imprinting, which suppresses transcription of these genes via epigenetic modification of the X-chromosome both via the transcription of the XIST gene, whose RNA product directly causes inactivation, and processes such as methylation, histone modification or chromatin remodelling that are differentially regulated between the sexes (Jazin and Cahill, 2010). Males inherit a maternal genomic imprint, while females inherit both the maternal and paternal imprints. This X-inactivation known as lyonisation results in females having a mosaic of cells with patches either expressing polymorphic alleles from the maternal or paternal chromosome, while males who have only one X-chromosome are always effectively homozygous. The differences in gene dosage result when these genes escape transcriptional inactivation (Reeve, 2009, Payer and Lee, 2008).

The basis for primary sexual differentiation resides in the expression of the Sry gene on the Y chromosome. Its protein, the testes-determining factor, regulates the transcription of other genes within the primordial gonads for their differentiation into testes instead of the ovaries. The developing gonads together with the adrenal glands are in turn vital for the synthesis and regulation of the sex hormones, which play an important role in the development of the brain both pre and post-natally (Goodfellow and Lovell-Badge, 1993).

Thus brain sex differences can be expected to originate from transcriptional differences in genes on the X-chromosome, Y-chromosome genes and hormonal effects. There have been some studies, mostly in vitro, demonstrating that functional and morphological differences exist between cultured neurons having an XX or XY complement of sex chromosomes even before hormonal regulation can diverge between the sexes (Arnold, 2004). Substantial evidence for a functional role of the complement of sex chromosomes, particularly the X-chromosome, in development and a number of secondary sexual characteristics such as brain dimorphism, although it is the SRY gene on the Y-

chromosome that is sex determining. For example, both individuals with Turner's syndrome or XO, having only one X-chromosome, and those with Klinefelter's syndrome or XXY are have morphological anomalies, with cognitive and psychiatric impairments despite having female and male primary sexual organs respectively (Good et al., 2003, van Rijn et al., 2006, Patwardhan et al., 2000). Additionally sexually dimorphic gene expression has been observed in the brain and other tissues prior to gonadal differentiation in the embryo with X-linked genes having higher expression within the brains of females regardless of inactivation status in mice (Dewing et al., 2003, Xu et al., 2002) and post-mortem human tissue (Vawter et al., 2004). It would also appear that a disproportionately high number of genes on the X-chromosome cause mental impairment when mutated as compared to the autosomes.

The Y-chromosome might be expected to have a more limited effect on sexual dimorphism due to only having 27 genes predicted to be unique to the human Y-chromosome (Arnold, 2004). In support of this, there have been relatively few studies demonstrating an influence of the Y-chromosome on the brain with one study showing no effect of Y-chromosome haplogroup on risk for autism (Jamain et al., 2002). Nonetheless, it appears that Sry itself is significantly expressed within the male brain and not the female brain and appears to influence the density of vasopressinergic fibres in the lateral septum in transgenic mouse models controlling for gonadal and chromosomal differences that could be imputed to impact on social behaviour (Arnold, 2004). Additionally, the sex-specific part of the Y-chromosome appears to be related to brain serotonin concentration (Tordjman et al., 1995). Furthermore, a number of genes on the Y-chromosome have been found to be expressed more highly in the brains of human males (Vawter et al., 2004). A recent study re-analysing gene expression data from various brain regions of several human embryos for sexual dimorphism found that the top 11 candidates for differential expression were on the region unique to Y-chromosome excluding the SRY gene (Reinius and Jazin, 0000).

The genetic contribution to regional brain differences has been described in part by brain mapping of X-chromosome deletions in Turner's syndrome, where it was found that the cerebellum, amygdala and orbitofrontal cortex are relatively larger with decreased X-

chromosome dose (Good et al., 2003). Testosterone appears to be an important part of the X-chromosome's contribution to brain development. Men with Klinefelter syndrome, a supernumerary X chromosome, have relatively less left temporal lobe grey matter and inferior verbal fluency that are both preserved if they are given testosterone supplementation from puberty (Patwardhan et al., 2000). This is a region of the brain shown to atrophy faster in males than females and to be smaller on the left compared to the right in men, but not in women (Cowell et al., 1994). Indeed, in adolescents aged 8-15 years, circulating testosterone is related positively to amygdala and hippocampal volumes and negatively to the grey matter volume of the left parietal cortex (Neufang et al., 2008). Administering testosterone to middle-aged healthy women increases amygdala and hypothalamic reactivity, as measured by fMRI (Hermans et al., 2008, van Honk et al., 2004) and modulates it in areas such as the orbito-frontal cortex involved in emotional regulation. Testosterone replacement in hypogonadal men increases cerebral perfusion of the midbrain, mid-cingulate and superior frontal gyri (Azad et al., 2003).

Sexual dimorphism in gene action is not however limited to the sex chromosomes. Sex chromosome or hormone-related factors may differentially regulate or induce epigenetic effects in genes on any of the autosomal chromosomes. One way to understand how these sexually dimorphic genes act can involve the investigation on the neural level of dimorphic gene associations

1.2.4.3 Sex hormones

1.2.4.3.1 Timescales of sex hormone action

While the sex chromosomes clearly have a role in sexual dimorphism in the brain, sex differences have been traditionally attributed to sex hormones and endocrinological influences are among the most studied in gender research. However authoritatively characterizing the hormonal influences on dimorphism is not straightforward as not only are androgens, oestrogens and progesterone present in both sexes, but their levels and

influences vary across life, in females across the menstrual cycle and in both sexes over the course of a single day. Such obstacles may account for the variability in findings from studies looking at hormones in behaviour and the subsequent difficulty of replication. One way to start to make sense of this complexity can be to consider the sex hormones in terms of the time scale over which they exert their influence. They are first commonly divided into activational and organizational influences. Activational effects are transient and often influence behaviour in specific contexts by modifying activity of the target cells. Activational effects are thus not present in the absence of the hormone causing them. Organisational effects occur through more lasting changes that occur in the brain and is widespread throughout development into adulthood. This too can differ depending on the structural changes that occur in the brain at different stages of life and I consider them during the periods of greatest sensitivity: in the fetus, the pubescent adolescent and the aging adult.

1.2.4.3.2 Activational effects

In this thesis, I have chosen not to deal specifically with activational effects due to their inherent lack of stability or ability to predict lasting traits. Therefore I do not investigate correlations with circulating levels of hormones or the influence of exogenous hormones despite the clear value to such approaches. Nonetheless, there is utility in discussing them briefly to provide a broader context for the body of work. In 1995, Van Goozen and colleagues performed longitudinal testing of 35 female-to-male and 15 male-to-female transsexuals. The study found that when androgens were administered to females over three months, they experienced an increase in aggression proneness, sexual arousability, and spatial ability with a decrease in verbal fluency. On the other hand when males were deprived of androgens, they experienced the converse (Van Goozen et al., 1995). While such observations could well be caused by short-term organisation due to exposure over three months or to the psychological or placebo effect of having a sex change, they are in broad agreement with the behavioral role of androgens. Hausmann in collaboration with that group went on to demonstrate that performance on the mental rotation test, an androgen-associated measure of spatial ability, varies significantly across the menstrual

cycle with high scores during the menstrual phase and low scores during the midluteal phase. Additionally, testosterone was positively correlated and oestradiol was negatively correlated with mental rotation performance (Hausmann et al., 2000). More recently, it has been shown that exogenous testosterone given to healthy females enhanced the neural responses of circuits activated in social aggression such as the amygdala and hypothalamus (Hermans et al., 2008) and emotional response to subconscious priming with fearful faces (van Honk et al., 2004, van Honk et al., 2005).

1.2.4.4 The fetus and neonate

In the male fetus, the developing testes secrete androgens as well as an anti-mullerian hormone that prevents the development of the primary genitalia of females. Testosterone, the primary androgen, is known to have masculinising effects across the body and may be converted to a much more active and specific form known as dihydrotestosterone by 5-alpha-reductase(Cooke et al., 1998). It is thought to exert what is known as an organisational effect in that it 'permanently influences the organisation of the brain a way that affects behaviour much later in life' during what has been called a 'critical period' (LeVay, 1994). Androgens are also produced by the adrenal glands and a syndrome known as congenital adrenal hyperplasia, which causes high early exposure to adrenal androgens through overstimulation by the hypothalamo-pituitary axis, can result in an intersex phenotype that has been shown to influence brain and behaviour prenatally. Androgen insensitivity is caused by a loss-of-function mutation in the androgen receptor or testicular feminisation, which can be complete or incomplete and results in a failure of formation of male genitalia despite an XY genotype. While females with one intact and one insensitive receptor have no difference in psychological outcomes from controls (Hines et al., 2003), males are demasculinised in their behaviour and in fact live like most females except for being sterile (Nelson, 2005, Carter, 2002). Placental androgen levels have been shown to relate to risk of autism and indirect markers such as the ratio of the length of the 2^{nd} and 4^{th} digits have been shown to be associated with a wide variety of sexually dimorphic aspects of behaviour(Lutchmaya et al., 2004).

In contrast, the ovaries of the female fetus are relatively inactive. The primary source of oestrogen and progesterone for both sexes is the pregnant mother. In males, a fair proportion of their oestrogen is produced by conversion from testosterone and is thought counter-intuitively to contribute to brain masculinisation. Compared to the androgens, the literature on the sexually dimorphic effects of these hormones on the developing fetus is sparse. Part of the lack of interest in oestrogen as a hormone in development may come from studies showing that that the development of the reproductive tract is independent of oestrogen. McEwen showed that rats express oestrogen receptor alpha in the hippocampus and the cerebral cortex. He also demonstrated a perinatal surge of aromatase expression for a brief period in late fetal and early postnatal development in mice particularly within the dorsal cingulate cortex. Finally he showed that there is sexually dimorphic expression of oestrogen receptor beta within the anteroventral periventricular nucleus of the rat preoptic area postnatally (McEwen, 2002, McEwen and Alves, 1999). The data on progesterone is yet sparser because like oestrogen most fetal and placental progesterone originates from the mother's ovaries. Yet even so, it is relatively neglected even in the adult literature as compared to oestrogen. It has been shown in vitro to regulate neurogenesis and proliferation of neural progenitors and so is likely to play an important trophic role overall during brain development, but it is unclear to what extent it may influence sexual dimorphism of the brain (Brinton et al., 2008, Wagner, 2006).

1.2.4.5 Puberty

Puberty is the transition from childhood to adulthood, when an individual becomes capable of sexually reproducing. It is characterised by elevated secretion of gonadal steroids. Individual variation in the response to these steroids can potentially cause the greatest range of difference in brain and behaviour. Puberty is initiated by the renewed activity of GnRH-secreting neurons in the hypothalamus that stimulate release of the gonadotrophins from the anterior pituitary, which in turn induces the release of the sex steroids from the gonads. The trigger to this initial activation of GnRH neurons is not fully understood, it is known to be

influenced by metabolic and energy balance as well as a number of candidate genes such as Kisspeptin and its receptor. This burst of hormones is thought to underlie most brain and behavioural changes that occur during puberty (Sisk and Foster, 2004). Although the primary result of puberty is sexual maturation, behavioural changes during puberty do not solely pertain to reproduction. Socially, there is a shift in interactions and general orientation from adults to peers with increased time spent with peers, less time spent playing and an elevation in conflict with parents-observations with parallels in non-human primates. Adolescents, especially males, also begin to exhibit increased aggression and risk-taking behaviour. It's thought that risk-taking and novelty-seeking behaviour has pertinence to adapting to and learning about the environment through increased exploration and potential pay-offs early in adult life (Spear, 2000). Adolescence is also generally associated with growth in cognitive ability, particularly in executive functions and age-related improvements have been observed in executive processes such as prospective memory, response inhibition, multi-tasking and perspective taking (Blakemore and Choudhury, 2006). Within the brain, adolescence is a period associated with thinning of the cortex especially in the frontal lobes associated with pruning, shrinking of the basal ganglia and growth of the amygdala and hippocampus(Giedd et al., 2006). Within the white matter, it has been demonstrated with DTI that radial diffusivity, that is negatively correlated with white matter integrity, decreases during puberty in the association, projection and interhemispheric tracts with later maturation of the superior longitudinal fasciculus in females and later maturation of the other tracts in males (Asato et al., 2010). It has also been shown that hormone levels modulate these processes as testosterone levels vary positively with the volume of the amygdala, hippocampus, diencephalon and negatively with left parietal volume, whileoestrogen levels vary positively with parahippocampal volume (Neufang et al., 2009). Overall white matter volume within the adolescent brain has also been found to increase more rapidly in male adolescents with increased testosterone levels and shorter AR CAG repeats(Perrin et al., 2008).

1.2.4.6 Aging

There is substantial evidence that these hormone-induced organisational changes occur throughout life and influence the trajectory of brain aging. For example, circulating sex hormone levels continue to influence brain structure post-adolescence. A study of adults in their mid-twenties found that left inferior frontal volume was associated with circulating oestradiol levels and inversely associated with circulating testosterone levels, while volume of the right temporal pole was associated with circulating progesterone (Witte et al., 2009). Late adulthood on the other hand, is a period associated with declining levels of the sex steroids. In women this is associated with a distinct period involving the cessation of menstruation and ovarian failure known as menopause where there is nearly complete loss of oestrogen, whereas in men the decline is more gradual. In women, menopause occurs around the age of 50 where the acute drop in oestrogen is frequently accompanied by depressed mood, sleep disturbance and various other physiological changes. The use of hormone replacement therapy has been shown to improve verbal memory, attention, processing speed and non-verbal reasoning. Older men with lower testosterone have worse performance in tests of verbal and spatial memory. Testosterone replacement therapy in hypogonadal men has been shown to improve mood in general energy, friendliness and lessen anger, nervousness and irritability (Morrison et al., 2006, Janowsky, 2006, Lamberts, 2002).

1.2.5 The candidate genetics approach

So how do these observed sex differences and the factors likely to influence them relate to the study of the brain. Sex differences pervade most aspects of brain and behaviour. Indeed the study of sex differences in the brain is as old as neuroscience itself, with early studies comparing brain weights in post-mortem brains and head measurements between men and women. With the advent of recent imaging techniques such as MRI, we are now able to non-invasively measure markers of brain function and structure in vivo. Coupled with computational neuroanatomical techniques, this has allowed the analysis and characterisation of large numbers of subjects, previously unheard of in conventional post-mortem studies and with a richness limited only by potential breadth of the techniques. Such large numbers have not only allowed robust characterisation of sex differences, but

have also amended themselves well to a broad range of studies. With the relative ease of determining sex, the measurement of sex differences has exploded with new but largely congruent findings with the development of each new technique. Such large numbers and the non-invasive nature of MRI lend themselves well to the genetic approach. Typical genetic studies require phenotypes that can be easily characterised in a large number of individuals for sufficient power as genetic effects tend to be subtle.

Yet how does the genetics approach aid the understanding of sex differences? What can it add to this established, yet still burgeoning field not previously possible? The physiological differences that may influence brain structure are as diverse and multitudinous as the differences themselves. Not only are men and women chromosomally different, but they also have a starkly dissimilar hormonal background. Circulating hormone levels may be different from actual brain concentrations, converted to other hormones at the target site and act on a number of different receptors with varying specificities for that hormone as opposed to other sex steroids. Thus a simple comparison between sexes can do little to dissect these individual confounding factors. The use of genetics to this end is ideal.

1.2.6 Molecular genetics

Gene polymorphism	Allele	Function	Expression	Validated?
PROGINS	320bp alu insertion. LD with V660L	Diminished function	Decreased expression	Yes, molecular genetic
AR(CAG)n	Exon 1, CAG repeat	Longer CAG tract causes decreased transactivation	Longer polyQ tract causes decreased expression	Yes, molecular genetic, disease
ESR1(TA)n	5' promoter region TA repeat	Short TA lower BMD	Longer TA repeats expected to relate to increased expression	Possibly in LD with functional
ESR2(CA)n	5'promoter intronal CA repeat	More androgen with short CA. Long CA, transcriptional binding, lower BMD.	Shorter CA repeats might relate to increased expression	Possibly in LD with functional
CYP19(TTTA)n	Intron 4 TTTA repeat	Men with high repeats, higher oestrogen/BMD. Women with high repeats, lower BMD.	High repeats might relate to increased aromatase expression	Possibly in LD with functional
CNTNAP2 rs3223460	TT risk homozygote confers autism risk vs TA/AA	Encodes caspr2. Polymorphism effect unknown	Unknown	
5-HTTLPR	Promoter region of serotonin transporter	Short (S) allele associated with lower serotonin levels	S allele associated with higher 5-HTT expression and thus reuptake of serotonin	Yes, molecular genetic
MAOA VNTR	30bp VNTR in promoter regions	Catabolism of serotonin and noradrenaline	3.5 and 4 repeats associated with high expression, 3 and 5 repeats with low expression of MAOA	Possibly in LD with functional

Table 1. 1 Summary of molecular genetics of candidate gene polymorphisms

One of the difficulties in attributing the neural effects of the sex hormones lies in their pleiotropic effects. A given sex hormone will act on several receptors both in the nucleus and outside of it and a degree of cross-receptor activity exists between receptors. Androgens are able to bind and cross-activate the oestrogen receptor and are converted to oestrogen by aromatase (CYP19), an enzyme expressed in several of the brain regions implicated. Thus some of the testosterone-associated correlations between brain structure and cognition may be mediated by the oestrogen receptor, while the oestrogen associations could be mediated by either of the oestrogen receptors. On the other hand, if one were to determine relatively subtle effects of individual receptors on sexual dimorphic traits, once could make a more discrete inference about that pathways effect less fettered by the confounds and interactions observed in gross comparisons between the sexes. Here several candidates have been identified, based on the breadth of literature implicating them as influences of individual variation in sex-relevant diseases and behaviours and the biological plausibility of their molecular genetic mechanisms.

Estrogen has a number of targets, which can act from the plasma membrane to various cytoplasmic organelles and the nucleus. It is thought that the oestrogen receptors are mostly localised in the cytoplasm as well as the nucleus. On bindingoestrogen, the cytoplasmic complex interacts with effector proteins to activate various kinases, which in turn modulate transcription factors. Within the nucleus, the ligand-bound ER interacts with Oestrogen responsive elements (EREs) that alter the expression of oestrogen-responsive genes (Nott et al., 2008).

The oestrogen receptor has 2 subtypes, alpha and beta, which are encoded by ESR1 and ESR2 respectively. Each subunit has differential affinity profiles across a range of ligands, although both bind oestradiol. The alpha subtype is well-known to influence the brain and is expressed in most regions with particularly high expression within the amygdala and hypothalamus(Laflamme et al., 1998). ESR1 contains a number of polymorphisms, of which the TA repeat 5’ of exon 1 is thought to influence expression levels. It is also in linkage disequilibrium with a number of other putative transcription binding sites. Evidence for the functionality of the polymorphism is further supported through its

association with a number of oestrogen-related clinical phenotypes such as female adult stature, bone mineral density, stroke and risk for ovarian and breast neoplasia (Molvarec et al., 2007, Ioannidis et al., 2004, Corbo et al., 2006, Holst et al., 2007, Slattery et al., 2007, Dunning et al., 2009). Longer TA repeats have been associated with increased anxiety in men and increased neuroticism, harm avoidance, psychoticism and irritability in both sexes (Westberg et al., 2003). Single-nucleotide polymorphisms of ESR1 have been shown to associate with premenstrual dysphoric disorder, a mood disorder linked to hormonal changes over the course of the menstrual cycle, as well as migraine and anorexia nervosa in women (Huo et al., 2007, Oterino et al., 2006, Westberg and Eriksson, 2008, Eastwood et al., 2002).

ESR2, encoding the beta subunit, spans about 254000 bp with 8 exons extending over about 140 Kbp. The 5' flanking region contains a number of regulatory elements including a CA repeat microsatellite polymorphism. This region is also relatively GC-rich and could be expected to be susceptible to methylation. Like ESR1 a number of ESR2 polymorphisms have been implicated in diseases related to old age and reproduction with associations in bone mineral density, cancers of the breast, prostate and endometrium, hypertension, pre-ecclampsia and cardiovascular risk (Eastwood et al., 2002, Ogawa et al., 2000, Setiawan et al., 2004, Rexrode et al., 2007, Maruyama et al., 2004). Additionally however, it has been associated with Alzheimer's disease, Parkinson's disease, chronic fatigue syndrome, anorexia nervosa, bulimia and risk for depression in an adolescent population (Westberg et al., 2004, Eastwood et al., 2002, Grans et al., 2007, Pirskanen et al., 2005, Tozzi and Bulik, 2003, Kravitz et al., 2006).

The progesterone receptor is encoded by the PR gene, which is located at 11q22. Two isoforms are known, the full length B isoform and the A isoform, which has a truncation at the N-terminal. Despite sharing several structural domains, they are distinct transcriptional factors with different response genes and physiologic effects. The A isoform transrepresses steroid hormone receptor activity when bound, while the B isoform functions as a transcriptional activator(Wagner, 2006, Brinton et al., 2008). The PR gene has a number of genetic variants. For example the PROGINS allele, which is explored in this thesis,

comprises a 306 bp ALU insertion within intron 7. The presence of the insertion causes decreased expression and response of the progesterone receptor and is linked to a valine-to leucine substitution and a synonymous polymorphism in exon 5. Another polymorphism at involving a G to A substitution at +331 of the PR promoter increases transcription and biases transcription towards the B isoform (Romano et al., 2007, Giangrande and McDonnell, 1999). Associations have been found between these polymorphisms and breast cancer, ovarian cancer, migraine-associated vertigo and panic in women (Ho et al., 2004, De Vivo et al., 2003, Lee et al., 2007, Modugno, 2004).

The androgen receptor mediates the action of the androgens within the nucleus, binding them preferentially and showing particular specificity for dihydrotestosterone. Its gene is located on the X-chromosome at q11-12 and has 8 exons. Like the progesterone receptor, the androgen receptor has A and B isoforms, which are both expressed across a variety of tissues. Longer repeats of the polymorphic polyglutamine tract in the N-terminal exon of AR inhibit its interaction with co-activators and the transcription of its gene to mRNA (Choong, 1996, Chamberlain et al., 1994, Beilin et al., 2000). Men who have the mutation with over 38 repeats manifest Kennedy's disease or spinobulbar muscular atrophy, while short repeats have been associated with risk for prostate cancer and benign prostatic hyperplasia. Similarly in women, AR CAG repeat length has been associated with androgen levels, bone mineral density and obesity as well as cancers of the breast and ovaries. AR CAG repeat length has been negatively associated with performance on the mini-mental state exam, digit symbol coding and Trails B, tests of general cognition and processing speed (Yaffe et al., 2003). It has also been positively correlated with some aspects of neuroticism and femininity (Jönsson et al., 2001, Loehlin et al., 1999) and negatively with psychoticism (Turakulov et al., 2004).

Aromatase is located within the electron transport chain of mitochondria and converts androgens into oestrogens. It is encoded by CYP19 on chromosome 15. Polymorphisms of CYP19 have been associated with sex hormone levels, bone density, and risk for cancer of the prostate and endometriosis as well as risk for Alzheimer's disease and depressive symptoms in women. One study reports an association between transsexualism and

CYP19, ESR2 and AR. Most of these studies report associations with the TTTA tetranucleotide repeat, a microsatellite polymorphism in intron 4 of CYP19. The polymorphism has a bimodal distribution with peaks at 7 and 11 repeats. Elderly men with more than 9 repeats have higher bone mineral density, lower bone turnover and higher serum estradiol than those with low repeats and from in vitro analysis higher repeats demonstrate higher aromatase activity, with faster metabolism of androstenedione to oestrogen (Zarrabeitia et al., 2004, Gennari et al., 2004). On the other hand in elderly women, the opposite was found to be true with carriers of the 7 repeat allele found to have lower risk of vertebral fracture and lower serum oestrogen levels (Dick et al., 2005).

The ratio of the length of the second (index)-to-fourth (ring) finger (2D:4D) was first proposed by Manning et al. (Manning et al., 1998) as a marker for *in utero* hormone levels. They found a stable sexually dimorphic pattern with males having a mean 2D:4D of 0.98 and females of 1.00, a dimorphism stable from the age of 2 years. They further found that in men, testosterone concentrations were negatively related to right hand 2D:4D, while in women and men, luteineising hormone, oestrogen and prolactin concentrations were positively correlated with 2D:4D. As a marker, 2D:4D has been associated with a diverse range of sexually dimorphic traits such as trait physical aggression in men (Bailey and Hurd, 2005), sexual orientation in men (Robinson and Manning, 2000), waist-to-hip ratio in women and male body mass index (Fink et al., 2003), apparent male facial dominance (Neave et al., 2003), reproductive success as measured by fertility in couples, likelihood of a woman being married and sperm viability in men (Manning et al., 2000), gender identity in females (Csathó et al., 2003), personality traits such as psychoticism, neuroticism and sensation-seeking in females (Austin et al., 2002) and risk for autism (Manning et al., 2001). 2D:4D has been shown in a twin study to have a heritability of roughly 66% (Paul et al., 2006) and so has a high genetic component, some of which may account for its numerous associations. In this thesis, 2D:4D is used as an additional marker in conjunction with the genetic markers to understand the molecular basis for stable sexual dimorphism in the brain.

While other hormones such as the gonadotrophin-releasing hormone, luteinising hormone, follicle-stimulating hormone and prolactin are also important in explaining the disparate hormonal background between males and females, they were not investigated due to a lack of good genetic candidates.

1.3 Scope

As discussed, sex hormones appear to act at different stages of life, often with varying effects. However the action of the receptors can be expected to be consistently modulated by genetic polymorphisms, such that genetic differences observed in the brain reflect the sum total of cumulative differential influences from embryogenesis and development into adolescence and adulthood. If as one might expect, these hormones act preferentially on certain regions or proceses at different stages, their 'mark' would still remain into adulthood. This thesis investigates these effects in conjunction with sexually dimorphic genetic effects or risk associations in three broad domains: Grey matter and overall compartmental volume; white matter and neural circuits; and finally behaviour focusing on personality. To attempt to dissect the entire body of sex differences into all possible factors would be both over-ambitious and outside the scope of the dataset which I have designed and collected, so this thesis seeks to exemplify some out of the broad range of questions to which this approach can be applied. To understand this, I first describe the nature of the dataset, the broad methods used and why they were chosen before going into the investigation of each domain in turn.

CHAPTER 2

DATA COLLECTION AND METHODOLOGY

2.1 Abstract

This chapter describes the scientific role of the field of imaging genetics and the rationale for taking the candidate gene approach in this thesis. Whereas in the previous chapter, the primary scientific question was posed, this chapter attempts to describe the means by which this question can be addressed. Thus genotyping methodologies are described and justified, and it is explained how this framework is used to investigate gene-behaviour associations. This chapter primarily deals with genetics and other general methods, while the imaging methods and other methods particularly relevant to certain results chapters are described later. I also describe how I set-up, designed and collected the data for the database, which I use in this thesis and my other studies.

2.2 The endophenotype approach in imaging genetics

Imaging genetics is a new and growing field that provides 'a strategy for mapping neural structure and activity as a function of genotype in living humans' (Meyer-Lindenberg and Weinberger, 2006). Not only has it enabled us to begin to understand the neural mechanisms underlying gene-behaviour or gene-disease associations thereby informing us about the psychological or medical phenomenon, but it has also given us insight about the systems level effects of genes themselves. While transgenic mouse models can provide relatively robust conclusions about gene function, they are limited in the range of phenotypes that can be covered as there are a number of important traits in such realms as

language, social cognition, higher level reasoning and personality in humans that are not amenable to study in rodents (Cacioppo and Berntson, 2002). Furthermore mouse models belie the function of a gene in humans due to the great divide in genetic and environmental background in which it is acting (Bucan and Abel, 2002). This thesis considers the effect of genetic variation on normal inter-individual variability, but in the awareness that this is one out of a number of possible approaches one could use to tackle the basic questions laid out in the introduction. To place a context on the potential epistemological significance of the chosen framework, these approaches are described and their complementary roles in elucidating a given phenomenon are explained.

Perhaps the earliest studies have employed imaging as one of several tools to characterise the phenotype of a disease-causing mutation. While MRI can be used to identify the abnormalities in the brain that result from established mutations such as in the mapping of preclinical Huntington's disease (Kloppel et al., 2009, Thieben et al., 2002) or the comparison of sporadic dystonia with familial and DYT1 dystonia (Draganski et al., 2009), it has also been used with novel genetics findings. For example, Vargha-Khadem et al followed up the linkage of SPCH1 to an inherited speech and language disorder with PET and quantitative MRI to identify structural and functional abnormalities (Vargha-Khadem et al., 1998). This approach has been further used to characterise subtypes of ataxia based on specific loci as in SCA11 (Worth et al., 1999), the effect of mutations in LRRK2 in Parkinson's disease (Khan et al., 2005), PCM1 mutation in schizophrenia susceptibility (Gurling et al., 2006) and many other disease conditions. Not only does it provide clues about possible mechanisms by which molecular disruption can lead to systems-level impairment, but it also provides functional evidence to back up the putative locus or mutation. Identifying imaging phenotypes has strengths beyond simple description as they can be used for subsequent mapping. Good et al described the differences between individuals with Turner's syndrome and controls using computational neuroanatomy and neuropsychological testing and then used deletion mapping with these neural and cognitive phenotypes to determine the critical region on the X-chromosome accounting for these traits (Good et al., 2003). Thus one approach has been to act as an auxillary to genetic studies identifying disease-causing mutations or loci by characterising neural correlates,

providing further evidence for the putative mutation and for closer mapping of a locus or gene.

In recent years, what is known as the endophenotype approach has become popular particularly in psychiatric genetics. Gottes and Gould describe endophenotypes as the 'measurable components unseen by the unaided eye along the pathway between disease and distal genotype' which can be 'neurophysiological, biochemical, endocrinological, neuroanatomical, cognitive or neuropsychological in nature'. They further outline the basic criteria for an endophenotype as the association with an illness in the population, heritability, state independence ie not requiring the illness to be present, cosegregation with the illness within a family and a higher prevalence in non-affected family members than the general population. Genes and polymorphisms that are implicated as functional and have established neural correlates are of further interest to cognitive neuroscientists and psychologists who are interested in the modulation of their cognitive processes by neurotransmitter pathways. By demonstrating individual differences between genotypes for a polymorphism, they can go beyond simply implicating regions to explore the realms of neuromodulation or can begin to unravel molecular influences on development and ageing.

While the endophenotype approach is very much disease-centred, parallels can be drawn between the study of a disease and the study of sex difference, thus suggesting that an analogous approach to that used to characterise polymorphisms conferring disease risk may be applied to the sex-related polymorphisms. Sex is a discrete entity: an individual can either be male or female just as someone either has a disease or does not. As described in the introduction sex, like most diseases, manifests as a complex phenotype with a range of biological factors that may influence it. Most sexually dimorphic neural phenotypes would fulfil some of the criteria for an endophenotype when taken in this sense. In addition to being associated with sex, many of these measures such as brain structure, personality and cognitive abilities such as arithmetic or processing speed have been demonstrated to be heritable. They vary to a high degree within sex and are thus not completely state-dependent. Perhaps more importantly, studies have reported genes and loci associated with these traits sometimes with gender-specificity(Fanous et al., 2002). Additionally, the

biological factors underlying sex have been well-described, so identification of candidates through the sex hormone pathways, sex chromosome linkage or associations with sexually-dimorphic traits and diseases, is made relatively straightforward.

Thus candidate genetics may be rationally applied to the general investigation of the systems level influences of molecular pathway and specifically to understanding the basis for sexual dimorphism in brain and behaviour. In this approach, I characterise normal genetic variation with previously identified polymorphisms and first determine if they are associated with a given sexually dimorphic trait. This allows an inference on which specific genes and therefore pathways are likely to be involved in the modulation of that trait. I go on to characterise the nature of this association by examining the relationships between associated factors and draw on these to suggest a mechanism by which these pathways may give rise to these sexual dimorphisms. To do this, it is necessary to understand how I set up the database and acquired the data, which I analysed for these associations.

2.3 Data collection

As mentioned, the initial motivation in setting up the database was to have a broad set of imaging, and personality measures against which genetic enquiry could be conducted and which could be used for subsequent recruitment for candidate studies in functional and structural imaging. It was also planned for the dataset to provide the background for a genome-wide association study. The study and recruitment procedures were approved by the local ICH-GOSH ethics committee. Total time to perform consent, screening, imaging and behavioural testing was about 3-4 hours per individual.

2.3.1 Recruitment

I contacted several thousand healthy individuals with normal structural brain scans at the Wellcome Centre for Neuroimaging and invited them back to the centre for follow-up scans, behavioural testing and to provide a blood sample for DNA extraction. All participants completed questionnaires providing information about medical or psychiatric conditions as well as their profession, net income, alcohol intake and smoking history. The MINI international neuropsychiatric interview is administered to screen for major Axis I psychiatric disorders in DSM-IV and ICD-10. Subsequently with specific collaborations, recruitment was also performed in tandem with recruitment for certain functional imaging studies where I obtained consent. At recruitment, each individual is assigned a study identification number, which was used to link across behavioural, DNA, imaging and personal information databases.

Gender	Male	Female	Total
Number	177	157	334

Age range	15-25	25-35	35-45	45-55	55-65	65-85	Total
Number	91	154	37	21	16	15	334

Scanner	Sonata 1.5T	Allegra 3.0T	Both	DTI	Total
Number	227	118	11	111	334

Table 2. 1 Tables showing demographic distribution of right-hand white population with genotypic data used in this thesis, T1-weighted 3D-MDEFT MRI scans of the specified sequence on either the Sonata 1.5T or Allegra 3.0T and fulfilling criteria for the absence of neurological or psychiatric conditions and for non-dependence or heavy regular useage of alcohol, recreational drugs or significant medication as screened.

Cognition	WTAR	QT	DSC	fDS	bDS	Arithmetic	Matrices	Computerised battery/ psychophysics
Number	199	201	237	197	231	206	300	394

Table 2. 2 Participants completing various tests of cognition. Wechsler Test of Adult Reading (WTAR)(Wechsler, 2001), Quick Test (QT)(Ammons and Ammons, 1962), Digit Symbol Coding (DSC), WAIS-III: Forward Digit Span (fDS), WAIS-III: Backward Digit Span (bDS), WAIS-III: Arithmetic, WAIS-III: Matrices (Wechsler, 1997). Computerised battery was designed by author and administered on Cogent, Matlab 6.5

Personality	STAI	NEO-PI-R	BSRI	KSP	SLS	CEI	BEQ	SHS	LOT-R
Number	160	160	160	146	134	134	134	134	134

Table 2. 3 Participants completing various tests of personality. State Trait Anxiety Inventory (STAI) (Spielberger et al., 1970), Revised NEO Personality Inventory (NEO-PI-R) (Costa and McCrea, 1992), Bem Sex Role Inventory (BSRI) (Bem, 1981), Karolinsa Scales of Personality (KSP) (Schalling and Edman, 1993), Satisfaction with Life Scale (Diener et al., 1985), Curiosity and Exploration Inventory (CEI) (Kashdan et al., 2004), Berkeley Expressivity Questionnaire (BEQ) (Gross and John, 1997), Subjective Happiness Scale (Lyubomirsky and Lepper, 1999), Life Orientation Test-Revised

Personality	TMMS	Empathy	TPQ	SSS	BIS/BAS	STAXI	SIRI	BFI	CFQ
Number	117	117	141	139	142	141	141	151	151

Table 2. 4 Participants completing various tests of personality. Trait Meta-Mood Scale (TMMS) (Salovey et al., 1995), Emotional empathy questionnaire (Empathy) (Mehrabian and Epstein, 1972), Tridimensional personality questionnaire (TPQ) (Cloninger et al., 1991), Sensation-Seeking Scale (Zuckerman and Neeb, 1979), Behavioural inhibition, behavioural activation Scales BIS/BAS (Carver and White, 1994), Stimulating and Instrumental Risk-taking Inventory (SIRI) (Zaleskiewicz, 2001), Big Five Inventory (John and Srivastava, 1999), Cognitive failures questionnaire (CFQ) (Broadbent et al., 1982)

Personality	AQ	MCSD	ATQ	MEQ	Handedness
Number	131	131	180	172	175

Table 2. 5 Participants completing various tests. Autism Quotient (AQ) (Baron-Cohen et al., 2001), Marlowe-Crowne Social Desirability Scale (MCSD) (Reynolds, 1982), Adult temperament questionnaire (Evans and Rothbart, 2007), Morningness Eveningness Questionnaire (MEQ) (Horne and Ostberg, 1976), Edinburgh Inventory (Handedness) (Oldfield, 1971)

2.3.2 Blood collection, storage and DNA extraction

In each individual, I performed venepuncture at the centre, collecting the blood in 2 X 6 mL EDTA tubes, which were temporarily stored at 4◦C before being brought to the research freezer in the Neurogenetics unit for storage at -80 ◦C. I performed extraction with the Qiagen DNA extraction kit together with a technologist at the Neurogenetics unit with a frequency between twice a week to every month in batches of 32 or 40, during which I entered each individual into the Neurogenetics database with their study number. This allowed me to use pre-existing infrastructure shared by the clinical service and clinical genetic researchers. I then quantified each sample for DNA concentration and quality on a Nanodrop 3300 and plated them into 96 Deepwell plates, normalising their concentrations to 0.100 mg/mL. Gel electrophoresis was also performed on random samples in order to ensure that DNA was intact and of good quality.

2.3.3 Image Acquisition

MRI was performed on a Siemens Sonata scanner operating at 1.5T and a Siemens Allegra scanner operating at 3.0T (Erlangen, Germany). The T1-weighted scans were acquired using a Modified Driven Equilibrium Fourier Transform sequence (MDEFT) (Deichmann et al, 2004). Diffusion tensor images (DTI) were acquired with 61 directions.

A subsample also underwent resting state functional imaging, participated in functional experiments investigating emotion, memory and social cognition and underwent a series of quantitative multi-spectral scans of T1, T2*, proton density and magnetization transfer imaging.

2.3.4 Behavioural testing

A number of standardized tests have been employed. These include measures of verbal ability, the Wechsler Test of Adult Reading and the Quick test. Several components of WAIS have been included, namely the digit symbol coding test and mental arithmetic. Forward and backward digit span and a computerized version of the matrices test for visuospatial reasoning are also measured.

I also designed and administered a number of cognitive tests to measure 2 choice and 8 choice reaction times as well as measures of conflict resolution using different variations of the Eriksson flanker test. Response inhibition is measured using a go-nogo task as well as a test estimating stop signal reaction time. Certainty equivalent, temporal discounting and ultimatum game behaviour are used to estimate economic preferences reflective of risk aversion, loss aversion and choice impulsivity.

Finally, self-report personality tests are administered online using questions from the NEO-FFI, the State-Trait Anxiety and Anger inventories, Trait Meta-Mood and Swedish university scales of personality, Adult temperament and tridimensional personality questionnaires.

In total, behavioural testing takes between 1.5 to 3 hours per participant.

2.4 Combining datasets

I created separate databases assigning subject identification numbers to plates and well positions, each online questionnaire entry, each MRI scan number and each set of cognitive and manually entered set of questionnaire and screening data. Individual folders for each subject ID were created for imaging and also for cognitive and psychophysics data. Individual analysis involved writing batch scripts on Matlab to extract necessary information for the specific analysis that is performed.

The design and development of the database represents only the first step in the project. Subsequent scientific enquiry required a range of genetic and image analysis techniques to be employed. I explain the theoretical basis for these techniques and justify the methodological choices that I have made.

2.5 The theoretical basis for genetics and genotyping

The double helix structure of the deoxyribonucleic acid (DNA) molecule was first proposed by Watson and Crick in 1953 (Watson and Crick, Watson and Crick, 1953). Beyond the description of a basic skeleton for the composition of the nucleic acid chain comprising repeating phosphate-deoxyribose sugar units, their structure introduced the concept of

unique base-pairing ‘in which the two chains are held together by purine and pyrimidine bases’, with adenine pairing with thymine, guanine pairing with cytosine and without any restriction on the base sequence on a chain, thus providing the basis for DNA as a heritable code. Technology in genetics has since been largely motivated by the goal of determining the nature of this base sequence at a locus or position and its distribution within a family or population, although in recent years there has been further interest in base modification such as in methylation and expression.

The Human Genome Project published the initial sequencing and analysis of the human genome in 2001 (Lander et al., 2001) that has provided a reference sequence against which specific genetic assays can be designed and the basis for a wide variety of bioinformatic investigations. It precedes the International HapMap Project that has developed and continues to work on a haplotype map of the human genome (HapMap) describing common genetic variation (Sachidanandam et al., 2001). Hapmap can be accessed via the web-based tool Haploview in order to access gene and fragment sequences as well as the common genetic variants, and their occurrence in different ethnic populations.

Genetic variation can be divided by scale into those involving a single nucleotide, the insertion or deletion of a sequence, variable number of tandem repeats and structural and chromosomal changes involving the amplification, deletion or translocation of larger spans of DNA. While the gold standard for determining the nature of a genetic variant involves sequencing a length of DNA including it, this can be costly and time consuming compared to the alternatives. In my development of genotyping protocols, I consider 2 types of polymorphisms that I have investigated in my candidate studies. The first class consists of variable number of tandem repeats [VNTR], and insertion/deletion [INDEL] polymorphisms. VNTRs consist of either microsatellites or minisatellites. In microsatellites there is typically anywhere between a 1-to-6-base pair sequence, typically dinucleotide or trinucleotide, that is repeated a number of times. Minisatellites may involve longer sequences that are repeated. These are typically genotyped by gene sizing. The second class are the single nucleotide polymorphisms where there is a single base pair differences between alleles in a population, while a broad range of approaches exist for

SNP genotyping polymerase chain reaction- restriction fragment length polymorphism [PCR-RFLP] is the simplest and most cost-effective with regards to single candidate polymorphism genotyping.

Amplification of the variation-containing sequence allows further genetic manipulations to be performed in order to detect the variant of interest with adequate sensitivity. The polymerase chain reaction (PCR) can amplify a given sequence through specific primers enclosing it from only a few copies of DNA by several orders of magnitude due to its exponential nature. First developed in 1984, PCR uses a heat-stable DNA polymerase originally from the bacterium Thermus aquaticus to replicate strands of DNA across thermal cycles. After an initial denaturation step that is often used to activate the reaction in hot-start PCR, there may be 20-40 cycles, each consisting of 3 steps. The first denaturation step involves separation of the double-stranded DNA into single strands by heating to around 95°C, thus making it accessible to the primers and taq polymerase. In the second annealing step, the temperature is lowered to a point where the primers can bind or 'anneal' to the single DNA strands to form a structure that bound by the taq polymerase. The optimum annealing temperature is dependent on the melting point of the primers used and a number of algorithms have been developed that can calculate the expected melting temperature given the sequence of the primer. The temperature is then heated to the optimum temperature for polymerization by taq polymerase. Each cycle under optimum and non-limiting conditions would thus double the number of amplicons or DNA fragments of interest, so if for example there were 35 cycles there would be 2^35, over 34 billion, times as many copies as originally in the template DNA. A final elongation is usually performed to complete the extension of any incomplete strands.

Restriction endonucleases, first isolated in the 1970, are enzymes that recognise specific nucleotide sequences, known as recognition sequences, for cleavage at a restriction site. A polymorphism with its neighbouring nucleotides can often form a recognition sequence with 1 allele and not the other such that digestion by restriction enzyme can be used to distinguish between alleles based on whether a fragment has been cut at that site. The fragments can then be separated by size using gel electrophoresis and subsequently

detected. In gel electrophoresis, DNA is separated using an electric current applied to a gel, in which the DNA has been placed. The gel usually consists of a matrix of cross-linked polymer most commonly agarose or polyacrylamide. When the electric field is applied across the gel, it exerts an electromotive force that moves the DNA, which at the commonly used pH is negatively charged due to the dissociated phosphate and acid groups, from the negative terminal or cathode to the positive terminal or anode. DNA from conventional PCR and restriction digests is linear and double-stranded and the speed it migrates through the gel matrix is approximately related to the inverse logarithm of its length. When DNA is run with a reference ladder containing fragments of known length, it is possible to estimate the absolute size of the molecule given the distance it has travelled.

A number of alternative and commonly used methods of genotyping exist, primarily for single nucleotide polymorphisms. These can be broadly classified according to the principle underlying the discernment between alleles into 3 other categories: Hybridisation with allele-specific probes, ligation with a specific sequence and primer extension. The decision to use PCR-RFLP rested on a number of factors. The first was the intention to genotype a small number of chosen polymorphisms in a relatively large number of individuals, thus high-throughput methods such as the use of DNA microarray chips would have been unsuitably expensive. The second was the need for high reliability and robustness across conditions, for this to be the case methods using ligation, allele-specific PCR and single base-pair extension tend to require optimisation of conditions in order to best distinguish between alleles and involve greater labour cost as #they have more steps than PCR-RFLP. Finally, allele-specific hybridisation methods while somewhat expensive to order per genotype would have been a suitable alternative as setting up the reaction is simple and each genotype can be used for a large number of individuals, however it requires real-time PCR, which was not available at the time of planning. Additionally, it is based on the relative size of 2 fluorescent peaks, which requires the judgement of a cut-off point.

PCR-RFLP, while certainly inexpensive due to the low cost of restriction enzymes and primers can be labour intensive at a large scale especially in the use of agarose gel

electrophoresis, requires manual calling, which can be difficult with smaller differences in fragment size or if a peak is weak. Human error is considered to be a major source of genotyping error, attributable for over 90% of the errors in one post-analysis of a study (Hoffman Amos; Pompanon 2005). To overcome this, the same fragment sizing methods employed to genotype microsatellite and insertion/deletion polymorphisms were used to detect the size of the digested fragments. These involved the use of a fluorescent primer, the design of the PCR such that detected fragments would be significantly larger than primer dimers and the additional denaturation step to generate single stranded DNA before being placed into the 3730xl DNA analyser. This allowed high-throughput genotyping to be performed with the additional advantages of sensitivity to low signal peaks, automatic size determination to within 2 base pairs and the robustness of all or none detection with PCR-RFLP provided complete digestion takes place. Thus the primary source of genotyping error lies in the failure of the PCR itself, a step present in most genotyping methods and an infrequent occurrence once optimised.

Both methods involve PCR-specific amplification of a fragment of interest, where relevant primers are designed for the genotyping assay. Primer design is performed by first finding the polymorphism on Hapmap (2007), downloading the flanking sequences in decorated FASTA format, and designing the primer using Primer3 (Rozen and Skaletsky, 2000). The primers are then blasted to ensure that they are unique to the sequence of interest by electronic PCR on the UCSC genome browser (Kuhn et al., 2006). This generates a predicted fragment of a given length that can be detected by various means. Optimisation of the PCR assay is performed by varying primer and template concentrations with the addition of Mg2+ or enhancer using a touch-down PCR protocol for the annealing temperature (Don et al., 1991). Annealing temperature is then optimised on a gradient block varying annealing temperatures 6 degrees above and below the predicted optimal annealing temperature. PCR-RFLP involves digestion of the PCR product by a relevant NEB enzyme, designed on NEB-cutter (Vincze et al., 2003).

Gene sizing is performed using an ABI 3730 system that detects the fragment length when labelled by a fluorescent primer. The labelled PCR amplicon is added to formamide

containing LIZ-500 size standard, denatured to single stranded DNA at 95 degrees for 5 minutes before snap-cooling and capillary electrophoresis.

2.6 Specific genotyping

Having explained the basis for my genotyping methodology, I provide the specific protocols used in this thesis for the determination of individual genotypes in each gene used.

2.6.1 Progesterone receptor, PROGINS

Genotyping was performed to detect the presence of the PROGINS allele through gene sizing for the presence of the 320 bp ALU insertion(Romano et al., 2007). The 175 bp or 495 bp PCR product was amplified by PCR with a HEX-labelled forward primer and an unlabelled backward primer:

PR1 HEX-5'-TTGAGTAAAGCCTCTAAAAT-3

PR2 5'-TTCTTGCTAAATGTCTGTT-3

PCR amplifications were performed in 25 uL reactions with denaturation at 94 ◦C

for 1 min, annealing at 60 ◦C for 2 min, polymerization at 72 ◦C for 2 min and repeated for 35 cycles. Thus the 495 bp PCR product reflects the presence of the PROGINS ALU insertion while the 175 bp product demonstrates its absence. 1uL of PCR product was added to 9uL of formamide with 0.3uL of LIZ-500 standard, denatured at 95°C for 5min and placed on ice. It was analysed on the ABI 3730 DNA sequencer equipped with Genescan (ABI, Warrington, UK) software.

2.6.2 AR exon 1 CAG repeat

For each individual, DNA was extracted from peripheral lymphocytes using standard techniques. A 370-450 bp fragment was amplified by PCR with a FAM-labelled forward primer

AR1 FAM-5'- GCCTGTTGAACTCTTCTGAGC-3'

AR2 5'- GCTGTGAAGGTTGCTGTTCCTC-3'

Amplification was performed in 33 cycles with a denaturation temperature of 95°C for 30s, an annealing temperature of 55°C for 30s and and extension temperature of 72°C for 30s, with a final extension of 72°C for 10min. 1uL of PCR product was added to 9uL of formamide with 0.3uL of LIZ-500 standard, denatured at 95°C for 5min and placed on ice. It was analysed on the ABI 3730 DNA sequencer equipped with Genescan (ABI, Warrington, UK) software.

2.6.3 ESR1 TA repeat

A 160-194 bp fragment was generated with a FAM-labeled forward primer:

ESR1F FAM-5'- -GACGCATGATATACTTCACC -3'

ESR1R 5'-GCAGAATCAAATATCCAGATG-3'

Amplification was performed in 28 cycles with a denaturation temperature of 95°C for 30s, an annealing temperature of 58°C for 30s and extension temperature of 72°C for 30s, with a final extension at 72°C for 10min. 1uL of PCR product was added to 9uL of formamide

with 0.3uL of LIZ-500 standard, denatured at 95°C for 5min and placed on ice. It was analysed on the ABI 3730 DNA sequencer equipped with Genescan (ABI, Warrington, UK) software.

2.6.4 ESR2 CA repeat rs3223460

A 147-187 bp fragment was generated with a FAM-labeled forward primer:

ESR2F HEX-5'- - GGTAAACCATGGTCTGTACC -3'

ESR2R 5'- AACAAAATGTTGAATGAGTGGG -3'

Amplification was performed in 35 cycles with a denaturation temperature of 95°C for 30s, an annealing temperature of 62°C for 45s and extension temperature of 72°C for 60s, with a final extension at 72°C for 10min. 1uL of PCR product was added to 9uL of formamide with 0.3uL of LIZ-500 standard, denatured at 95°C for 5min and placed on ice. It was analysed on the ABI 3730 DNA sequencer equipped with Genescan (ABI, Warrington, UK) software.

2.6.5 CYP19 TTTA repeat

For each individual, DNA was extracted from peripheral lymphocytes using standard techniques. A 370-450 bp fragment was amplified by PCR with a FAM-labelled forward primer

CYP19_F FAM-5'- GCAGGTACTTAGTTAGCTAC -3'

CYP19_R 5'- TTACAGTGAGCCAAGGTCGT -3'

Amplification was performed in 35 cycles with a denaturation temperature of 95°C for 30s, an annealing temperature of 55°C for 30s and and extension temperature of 72°C for 45s, with a final extension of 72°C for 10min. 1uL of PCR product was added to 9uL of formamide with 0.3uL of LIZ-500 standard, denatured at 95°C for 5min and placed on ice. It was analysed on the ABI 3730 DNA sequencer equipped with Genescan (ABI, Warrington, UK) software.

2.6.6 CNTNAP2 rs7794745

DNA was extracted from the subjects' blood samples and genotyped by PCR-RFLP. Primers were designed on Primer3 software (Rosen and Skaletsky 2000) from a DNA region 500bp flanking rs7794745, found and blasted by electronic PCR on the UCSC genome browser NCBI build 36.1 (Karolchik et al. 2008). The restriction enzyme Tsp509I (New England Biolabs) was selected as the enzyme with differential cleavage at rs7794745 on NEBcutter (Vincze et al. 2003) such that a sense strand T-allele produces a fragment of 214 bp and 57 bp (not fluorescent), and an A-allele produces a fragment of 271 bp.

Forward: 5' HEX- GGCCCTTGCATATAGTTCCA- 3'

Backward: 5'- CCAACAGTGCCTTGTGTCA- 3'

Genotyping was performed through polymerase chain reaction (PCR) followed by restriction digest and subsequent capillary electrophoresis. PCR with Taq polymerase (Molzym) involved initial denaturation at 94 ^{o}C for 5 minutes, followed by 35 cycles of denaturation at 94 ^{o}C for 15 seconds, annealing at 60 ^{o}C for 30 seconds and elongation at 72^{o}C for 45 seconds, followed by 72 ^{o}C for 7 minutes. Restriction digest was performed using 1U of Tsp509I, and 2 uL PCR product in 4 uL total volume at 65^{o}C for 5 hrs. This was heat denatured to single-stranded fragments in formamide and run with a ROX500

ladder on a 3730xl DNA Analyser (Applied Biosystems). Individual genotypes were called according to peak size on GeneMapper software version 4.0 by 2 independent raters into those homozygous for the T allele, heterozygous for the T and A alleles and homozygous for the A allele.

2.6.7 5-HTTLPR

A protocol was used as is described in Roiser et al and Strange et al (Roiser et al., 2009, Strange et al., 2008) and modified from Furlong et al (Furlong et al., 1998) to genotype the 5-HTTLPR. Briefly this involved restriction enzyme digests with HpaII (Wendland et al., 2006). PCR was performed across the 5-HTT promoter insertion/deletion using primers (Heils et al., 1996) (Invitrogen):

stpr5: 5_ GGC GTT GCC GCT CTG AAT GC 3

stpr3: 5_ GAG GGA CTG AGC TGG ACA ACC AC 3

This involved initial denaturation for 5 min, followed by 35 cycles of 95°C for 0.5 min, 61°C for 0.5 min, 72°C for 0.5 min, and final extension at 72°C for 10 min. The 25 uL reaction comprised 1 uL DNA, 250_M dNTPs, 1 uL enhancer (Molzym),

1 uL PCR buffer (Molzym), and 0.625 units *Taq* polymerase (Molzym). The PCR product (10 uL) was loaded on 1% UltraPure agarose gel stained with ethidium bromide and run for 1 h at 80 *V* in Tris-borate-EDTA buffer (TBE) with a 100 bp ladder (Invitrogen) typing the 528 bp and 484 bp products as the l and s alleles, respectively. PCR product (10 uL) was digested by HpaII (5 U; New England Biolabs) in a 20 uL reaction assay containing 1 uL NEBuffer 1 and 1 uL bovine serum albumin at 37°C for 3 h. Restriction enzyme assay solution (18 _l) were loaded on 4% UltraPure agarose gel and run for 2 h at 120 *V* in TBE with a 25 bp ladder (Invitrogen), producing 298, 126, and 62 bp fragments for the sa allele;

167, 131, 126, and 62 bp fragments for the sg allele; 341, 126, and 62 bp fragments for the la allele; and 174, 167, 126, and 62 bp fragments for the lg allele. Genotypes were subsequently revalidated using a FAM-labelled forward primer under identical PCR and restriction digest conditions. The digest was heat denatured to single-stranded fragments in formamide and run with a ROX500 ladder on a 3730xl DNA Analyser (Applied Biosystems). Individual genotypes were called according to peak size on GeneMapper software version 4.0.

2.6.8 MAOA VNTR

Samples were genotyped by polymerase chain reaction (PCR) amplification using the following primers:

5'FAM-ACAGCCTGACCGTGGAGAAG-3'

5'-GAACGGACGCTCCATTCGGA-3'

The first primer was fluorescently labelled with FAM on its 5' end and the primer pair was blasted with the in-silico PCR application on the UCSC genome browser (Kent *et al.*, 2002). Amplification was performed in a final volume of 25μL, 25ng genomic DNA, 200μM dNTP, 10pmol of each primer and 0.25 U Taq DNA polymerase (Molzym, Germany) in the standard buffer at 1.5 mM MgCl2. Cycling was optimised and was performed at 30s at 92 ºC denaturation, 1min at 62 ºC annealing and 1min at 72ºC extension for 35 cycles with a final extension of 72ºC for 7min (Sabol *et al.*, 1998). In each well, 2μL of the PCR product was added to 2.5μL of formamide and 0.5μL of 500XL ROX size standard before denaturation for 5min at 95 ºC and being placed on ice. The size of the PCR amplicon in the final mixture was determined by GeneScan on the 3730xl and analysed by GeneMapper (Applied Biosystems, UK) by an independent blind investigator.

	Normal homozygote	Carriers	Risk homozygote
MAOA VNTR low-expressing	127	58	44
OPRM1 A118G	237	78	5
5HTTLPR short allele	89	108	59
PROGINS	297	88	
BDNF val66met	27	106	91
DAT-9R	148	139	15
Kibra rs17070145 T allele	79	73	
DRD2 Taq1A	217	137	13
Aromatase CYP19 TTTA >9R	126	179	40
CNTNAP2 rs7794745 Tsp509I	143	190	47
CNTNAP2 rs2710102 BSOBI	67	84	44
ABAT	248	106	12

Table 2. 6 Allele frequencies of candidate gene single nucleotide polymorphisms

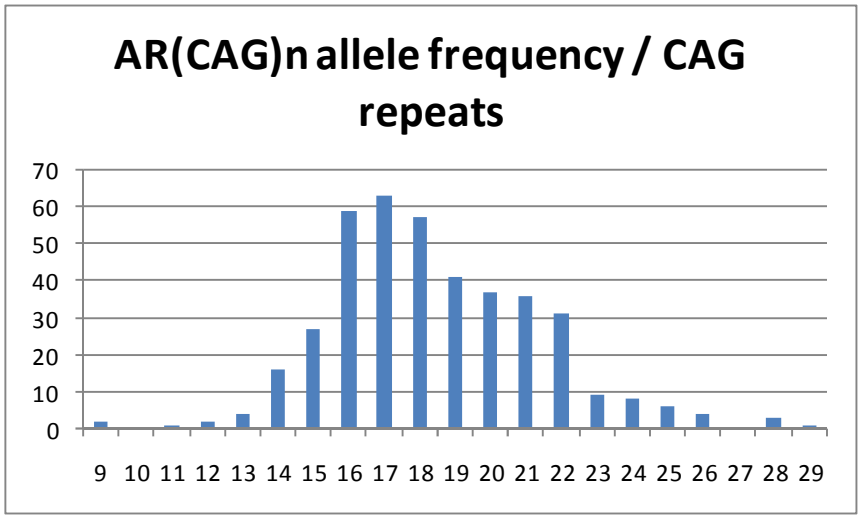


Figure 2. 1 AR(CAG)n allele frequencies

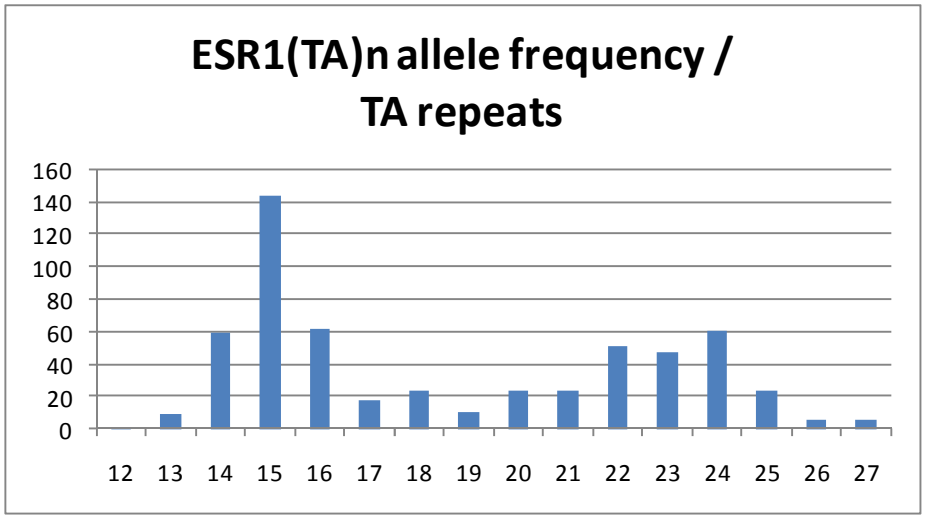


Table 2. 7 ESR1(TA)n allele frequencies

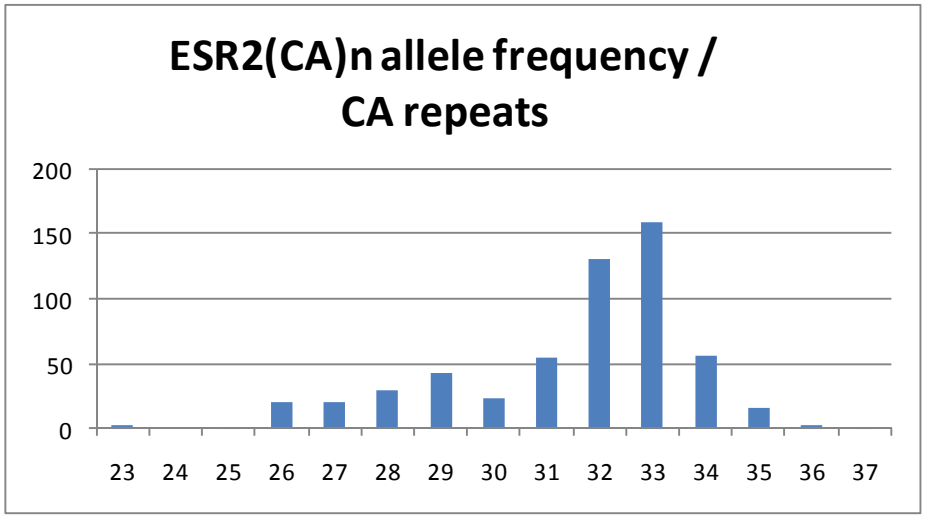


Table 2. 8 ESR2(CA)n allele frequencies

2.7 Magnetic Resonance Imaging (MRI)

In addition to the use of genotyping to determine individual genetic information, MRI was used to provide measurements of various aspects of the brain. The technique is based on the use of magnetic fields by Rabi to measure atomic spectra and the discovery by Bloch and Purcell that when certain nuclei are placed in a magnetic field they could absorb radiofrequency waves and re-emit them at a frequency determined by the Larmor equation known as Nuclear Magnetic Resonance, for which Nobel prizes were awarded in 1944 and 1952 respectively. Lauterbur subsequently invented a means of forming an image by inducing local interactions with NMR in 1973 using magnetic gradients, where he imaged 2 test-tubes of water, while Mansfield worked out a means of mathematically analysing the signals to form an image.

In this thesis, MRI was used for the approximate measurement of T1 and diffusion across the brain. Nuclei with an odd number of nucleons possess net spin, a property that arises from the individual spins of component nucleons. In MRI, typically the spin of hydrogen nuclei in water is used for measurement by NMR. When objects are placed within a strong magnetic field, these nuclei align in the direction of the magnetic field and can absorb a photon at what is known as the Lamor frequency, which depends on the product of the particle's gyromagnetic ratio and the magnetic field that it is in. The absorption of a photon causes a transition from a low energy to high energy state. Thus the emission of a radiofrequency wave at the appropriate Lamor frequency causes a shift in the proportion of hydrogen nuclei in the high energy state. This can be also be understood as a shift in the net magnetisation vector that is defined by this proportion. This magnetisation vector lies along the direction of the applied magnetic field or Z axis at equilibrium. With the absorption of the radiofrequency wave, this shifts from longitudinal to the transverse direction as the spin packet precesses around the axis of the applied magnetic field at an increased angle.

The T1 of a tissue, or its spin-lattice relaxation time, is defined as the time taken to reduce the difference between its longitudinal magnetisation and its equilibrium value by a factor of e. The net magnetisation between spin packets also begin to dephase as the transverse magnetisation relaxes differently due to local difference in magnetic field. The time taken to return to the equilibrium of transverse magnetisation is known as the spin-spin relaxation time or T2. Different tissues in the brain have different T1 and T2 values as the water-bound hydrogen atoms experience differing local magnetic milieu and due to the presence of and interactions with other nuclei and carbon-bound hydrogen atoms. Thus measurement of T1 in different brain tissues having differing compositions such as white matter, which contains more fat, versus grey matter, which contains more water and protein, produces intensity contrasts that allow us to distinguish these tissues.

MRI is able to produce an image or individual measurements of various locations by exerting a unique magnetic field strength and thus a different Lamor frequency in each part of the excited slice through the use of linear magnetic field gradients in the x or frequency-encoding, where Lamor frequency at that location reflects location along that axis, and y or phase-encoding, where a shift in the phase angle is induced by the gradient, directions perpendicular to the primary magnetic field. The slice is selectively excited by using an excitation pulse that has a frequency bandwidth covering the Lamor frequency of hydrogen nuclei at the desired slice along the z direction. (Hornak, McRobbie et al., 2003)

2.8 Image Acquisition

MRI was performed on a Siemens Sonata scanner operating at 1.5T and a Siemens Allegra scanner operating at 3.0T (Erlangen, Germany). For VBM, a three-dimensional structural MRI scan was acquired from each subject using a T1-weighted MDEFT sequence (176 slices, 1mm isotropic, no interslice gap, sagittal acquisition, FoV 224x256 mm, matrix 224 x 256, fat saturation) (Deichmann et al, 2004). Sequence parameters were optimised to maximise contrast to noise ratio between grey and white matter, while decreasing artefacts such as drop-out.

For DTI, the DW images were acquired by applying gradients in 61 directions with a b-value of 1000 s/mm^2 as well as 7 with a low b-value of 100 s/mm^2 (TE = 90 ms, isotropic resolution = 2.3 mm, slices = 60, matrix size = 96× 96, field of view = 220 mm, twice refocused diffusion encoding) (Nagy et al., 2007).

2.9 Image preprocessing

2.9.1 VBM methodology

Image preprocessing can significantly influence eventual findings. In comparing two samples for example, a technique such as voxel-based morphometry determines the locations of statistical difference between the two groups. However in addition to a true biological difference, significance can arise when there are systemic differences in preprocessing or in the physical acquisition of the images. A number of approaches have been developed to try to get around this based on the theoretical and practical constraints of the standard preprocessing. These have included an automatic brain extraction, the use of customised templates and priors. The first two features attempt to improve tissue classification, while the third enables inference to be made about regional tissue volumes, as opposed to local composition. The simple preprocessing procedure include misclassification of blood containing compartments such as the dural venous sinuses and diploic spaces, scalp fat, and parts of the petrous bone. Optimised VBM weights the registration to align grey matter, the subject of comparison, and these voxels are removed with an automated brain extraction using erosion and dilation. Comparisons have been made of different VBM preprocessing procedures on SPM2 for group differences between the healthy elderly and Alzheimer's disease patients from the standard protocol and the authors showed that optimised VBM and customised templates/priors to be successively more sensitive to the group effect, while the addition of a re-initialisation step removed the ventricular and cerebellar differences, which they considered biologically implausible (Senjem et al., 2005, Keller et al., 2004).

Segmentation on SPM5 has been optimised through a procedure known as unified segmentation . It “enables image registration, tissue classification, and bias correction to be combined within the same generative model” with a single log-likelihood objective function. After an initial affine registration, an iterative procedure is performed involving alternating tissue classification, registration and parameterisation of image intensity nonuniformity in a circular fashion to optimize the objective function. Tissue classification is performed by first characterizing the intensity distribution of each class by a fit to a mixture of Gaussians, with the voxels making up the distribution first defined by a Bayesian weighting of voxels lying within a prior tissue probability map. Within the cost function, the entropy of the histogram of log-transformed intensities is defined so when this is minimised there is a uniform overall bias field. The prior tissue probability maps are then registered to the individual to optimise the objective function, while the algorithm is generalisable across a family of registration methods, the implementation in SPM5 parameterises the deformations by a linear combination of cosine transform bases (Ashburner and Friston, 1999).

Additionally there is a regularisation term in the cost function that penalises large values for intensity uniformity so that the field does not also model inherent tissue properties. The unified segmentation procedure, while taking into account most of the considerations used in the specialised preprocessing pipelines mentioned above, does suffer from its own limitations. Firstly, it is computationally more intensive as it uses a more complex cost function with more parameters to compute at a given time. Secondly, as an iterative procedure –Iterated Conditional Modes- it only computes a locally optimal solution, thus poor starting parameters often due to the initial affine registration, which is based on a sparse set of features, can cause errors in the segmentation procedure. Finally, the procedure makes use of only three prior tissue classes, which as a subjective definition of intensity clusters are not sufficient to describe the tissues that make up the brain; within a given tissue class such as grey matter there are regional intensity differences, while a number of tissue classes such as partial volume and non-brain tissue are inadequately modelled.

The registration procedure performed in the unified segmentation procedure or provided in SPM suffers from a number of limitations. Firstly, only about a thousand deformation parameters are used to perform the mapping from the individual to a common template. Secondly, the mapping that is calculated has not been formatted in such a way that it is invertible; the forward mapping is not a one-to-one smooth and continuous mapping. While this is often approached within a small deformation model when displacements are small, it is inadequate for larger deformations which occur when a fine mapping is required that rigorously minimised residuals. These limitations can be overcome by a large-deformation or diffeomorphic framework (Miller, 2004), however there remains a trade-off between high dimensional and accurate algorithms and computational requirements. SPM uses a toolbox called DARTEL, or Diffeomorphic Anatomical Registration using Exponentiated Lie algebra, that allow diffeomorphic deformations that are relatively rapidly computed (Ashburner, 2007). While it less flexible than other diffeomorphic frameworks in that it uses a fixed rather than a variable velocity model, it allows invertible high-dimensional registration to be performed with increased sensitivity and spatial homology in VBM comparisons. In the current framework, segmented images are first averaged to form templates to which individual maps are warped. These are in turn used to generate another set of more spatially precise templates in an iterative manner with decreasing contraints on the degree of the deformation.

Another major factor influencing VBM analysis is the sensitivity and uniformity of the scanning parameters. Different field strength and sequences can be more or less subject to geometric distortions and inhomogeneity that may not be completed corrected for by image processing. Additionally, scanner drift over time can be a major factor if date of scan is a significant confound. In the current dataset, phantoms are used to ensure uniformity of the scanner over time and any changes to sequences are modelled within the statistical analysis. Collaborating with Cynthia Stonnington and others, I consider this question in terms of testing the influence of multiple scanners on VBM analysis (Stonnington et al., 2008b). The dataset consisted of 136 subjects, 62 with Alzheimer's disease and 74 cognitively normal elderly individuals acquired on six different scanners with multiple upgrades. With the above algorithm, I tested for scanner differences, group differences and an interaction of scanner with disease across the different scanners and found empirically that the

preprocessing algorithm I employ in this study is robust to the effect of scanner differences and that it does not significantly affect the analysis of differences between diseased and control individuals. While validating the preprocessing approach that I use here, it does not address the question of scanner sensitivity to regional differences, which is beyond the scope of this report. While increased scanner sensitivity is likely to decrease false negatives, there is no evidence that scanning parameters when adequately modelled within the analysis can generate false positives. Thus any positive findings are unlikely to be invalidated in this approach, while the converse is true with a failure to replicate a regional finding that might have been discovered elsewhere.

Scanner field and sequence can be a significant confound in any analysis. The two main ways that field strength, scanner and sequence present as confounds are in spatial or geometric distortion and in signal intensity and sensitivity. There is the potential for geometric distortion to cause false localisation of an effect, although the shift has been shown empirically to constitute a few millimetres, and accountable by the warping algorithm and smoothing during preprocessing. Geometric distortion as with signal intensity can lead to changes in the intensity of the modulated probability map by changes to the estimated volume change in the Jacobian determinant and the probability of the tissue class at affected voxels. Such scanner influences can in turn be linear, independent from the factor of interest, and non-linear, where there is an interaction with a measure of interest. The paper cited (Stonnington et al., 2008a) demonstrates that such non-linear effects are generally not substantial as interindividual effects are relatively subtle although scanner effects are large (so scanner effects are equal between populations) and that it would be reasonable to model scanner as a covariate (where linear confounds of scanner are completely accounted for). Nonetheless all analyses modelled scanner type as a condition within the general linear analysis. This effectively models the statistical question in each population (in each scanner) and detects effects generalisable across [scanner] populations and is thus equivalent to a meta-analysis taking into account non-linear effects of scanner.

Voxel-based morphometry [VBM] makes inferences voxel by voxel about local compositions of different tissue types across and between populations while discounting, at a given spatial level, positional and global volume differences. As compared to

deformation-based morphometry [DBM], where volumetric inference is made purely on the basis of the estimated volumetric difference from deformation parameters in the warp, VBM attempts to take into account residual differences between individuals not accounted for by the warp in some meaningful way. Wright et al first used VBM in 1995 to compare schizophrenia patients with controls. It has been subsequently developed within Statistical Parametric Mapping [SPM] software (Ashburner and Friston, 2001). Unmodulated VBM theoretically discounts global shape change and a smooth spatial normalisation places focal differences across individuals in the same stereotaxic space for comparison. This is criticised by Bookstein as unreliable due to the lack of interchangeability between global shape change and residuals and thus its lack of meaning as a volumetric measure (Ashburner and Friston, 2001). Indeed unmodulated VBM has largely fallen into disuse in recent years with the exception of use in empirical approaches such as brain prediction and classification. With 'modulation', volume changes during registration are corrected with the Jacobian determinant of the warp allowing more meaningful inferences to be made about regional volumes as 'tissue densities', summing the differences due to both the warp and its residual (Ashburner et al., 1998). Although globally, this has been shown to completely account for individual volumetric differences, this too has its limitations as it can be influenced by the accuracy and estimated tissue probabilities of the segmentation procedure and voxel-wise comparisons may not always compare homologous structures with errors in registration. Empirically the segmentation procedure has been optimized through a number of difference preprocessing methods, but remains an important issue. The registration error is broadly corrected for through smoothing and the naturally smooth nature of anatomical differences as they arise during development, but can potentially cause registration-dependent differences and incorrect spatial attribution of a volumetric difference.

Except the sections looking for global effects across the entire brain, most analyses were targeted and hypothesis-driven so correcting for multiple comparisons across analyses would be neither meaningful nor necessary. The goal here was different from that of a conventional genetics study where statistics are used to implicate a subset of many genes through an association. Instead analyses were both constrained to known and experimentally confirmed sexually dimorphic traits seeking to compare the differential contributions of molecular pathways on the basis of the influence of gene variants on the

population variance of those phenotypes. Sexually dimorphic traits are also correlated by sex within a population and if influenced by the same molecular factors are likely to be correlated across sex as well, thus analyses are also not independent further complicating attempts at correction for multiple comparisons. Additionally many analyses such as interactions and regional volumetric analysis were further characterisations of initial findings asking more refined questions of a basic finding. Nonetheless, asking several independent questions will inevitably result in false positives. This is true regardless of whether those questions are asked of the same or independent datasets especially with the bias towards publication of positive results. Thus when a particular gene or phenotype is extremely popular within the field, a number of reported studies will be false. This issue could be dealt with in a number of ways. Firstly, an approach could be taken as suggested to modify statistical thresholds or correct for multiple comparisons despite having clearly distinct main hypotheses. This approach can be problematic as some of the phenotypes are likely related in a way that is useful to defining the overall contribution of a pathway to sexual dimorphism and there is no accurate way to model these dependencies in the context of a multiple comparison. Genetic association studies are somewhat illustrative of issues with this approach where high rates of non-replication occur even though the most stringent of statistical corrections are employed, while candidates lower down the statistical ladder are eventually replicated and there is no consensus on the best statistical approach to multiple testing. Each section of the analysis of specific phenotypes (non-global or voxel-level phenotypes) considers multiple comparisons across genotype within the section using Bonferroni correction and places an * on results surviving corrections.

The second approach is replication of a result and this replication can either be direct and statistically independent or biological. Within my dataset, main results such as the association of the hypothalamus, and temporal and orbitofrontal cortex GM volume with AR(CAG)n and global brain volume with PROGINS were replicated between scanners and between the first 96 (first plate run) and subsequently genotyped individuals. Publication of the results will also allow others to replicate the results of the study. More importantly to the drawing of broad scientific conclusions is biological replication where studies of the same pathway or broad association are considered together to arrive at theories and mechanisms that can then be tested. Crucially, the discussion juxtaposes specific results from my experiments and analyses with those from other studies looking at hormonal

contributions to the brain pointing out areas of convergence where evidence for a broader conclusion is built and considering why they might diverge both in the statistical and biological contexts.

Family-wise error correction on the cluster and voxel level was performed for multiple corrections across the entire brain, thresholded at $p<0.05$ and this approach has been used for all reported regions in the text of the thesis. The protocol for performing this was based on the current consensus within the field which is to first perform uncorrected voxel-level thresholding at $p<0.001$ and to extract clusters or voxels achieving significance at $p<0.05$ fwe-corrected. This was performed using the non-stationarity toolbox for SPM (Hayasaka et al., 2004). Often clusters surviving correction will not have any voxels surviving correction and as such it is standard and correct to display figures at $p<0.001$ uncorrected. Figures were used for display purposes and as such when effects were highly significant as with the main effect of the AR CAG polymorphism, voxel thresholds of $p<0.05$ fwe-corrected are used so that individual regions can be distinguished by eye. This does not change the results reported.

2.9.2 VBM procedure

VBM was performed on grey and white matter volumes as well as comparison of FA using the SPM5 software (Wellcome Trust Centre for Neuroimaging, London, UK, http://www.fil.ion.ucl.ac.uk/spm/) run on Matlab version 7.0. Grey and white matter images were created in native space for each individual using unified segmentation to partition each T1 image into tissue classes of grey matter, white matter, CSF, and non-brain voxels (Ashburner and Friston 2005). The resulting segmented tissue probability images were used to estimate a nonlinear warp to a population template generated from the complete dataset using DARTEL (Diffeomorphic Anatomical Registration Through Exponentiated Lie algebra). DARTEL aims to achieve a more precise inter-subject alignment, increasing both sensitivity to and localization of anatomical differences (Ashburner 2007). The MNI single subject T1-weighted atlas was also warped to the population template and a composite deformation of each subject into MNI space was used to generate modulated

grey and white matter images, which were then smoothed by convolving with an 8mm FWHM Gaussian kernel.

2.9.3 Procedure for voxel-wise FA comparison

Diffusion tensor images were processed using FSL software (FMRIB Software Library, FMRIB, Oxford, UK) (Smith et al. 2004). The 7 low b100 images were coregistered to create a mean image to which all 68 images DW images were coregistered by an affine transform and eddy-corrected using FLIRT and FDT (FMRIB Linear Image Registration and Diffusion Toolbox). A brain mask was created from the mean b100 image and FDT was used to fit a tensor model to compute FA, MD, axial diffusivity and radial diffusivity. Preparation for FA VBM was performed using a modification of the Tract-based spatial statistics procedure (Behrens et al. 2003, Woolrich et al. 2009). Briefly, TBSS was used to run the initial preprocessing and nonlinear registration to the MNI-DTI template, also known as the FMRIB50_FA image in standard space, (Smith et al. 2006) as well as the creation of the mean FA and skeleton images derived from the FMRIB50_FA image. The skeleton images were thresholded at 0.2 and used as an extrinsic mask in subsequent FA VBM analysis on SPM5 utilising the smoothed warped FA images. This allowed a direct comparison between WM VBM and FA VBM results.

2.10 Relevant co-authored papers

Stonnington, C. M., **Tan, G**., Kloppel, S., Chu, C., Draganski, B., Jack, C. R., Jr., Chen, K., Ashburner, J. & Frackowiak, R. S. 2008. Interpreting scan data acquired from multiple scanners: a study with Alzheimer's disease. *Neuroimage.*, 39, 1180. (Stonnington et al., 2008b)

Kloppel, S., Chu, C., **Tan, G**., Draganski, B., Johnson, H., Paulsen, J., Kienzle, W., Tabrizi, S., Ashburner, J. & Frackowiak, R. 2009. Automatic detection of preclinical neurodegeneration: Presymptomatic Huntington disease. *Neurology*, 72, 426. (Kloppel et al., 2009)

CHAPTER 3

BRAIN STRUCTURE: SEX DIFFERENCES IN TISSUE COMPARTMENT AND REGIONAL GREY MATTER VOLUME

3.1 Abstract

This chapter considers the contribution of specific genes in sex hormone pathways to individual variation in sexually dimorphic measures in brain volume and regional grey matter distribution. The chapter comprises 3 sections. In the first section, the contribution to known sex differences in overall grey matter, white matter and whole brain volume by the PROGINS polymorphism of the gene for the progesterone receptor is characterised. The second section compares the individual contributions of polymorphisms in the genes for the androgen receptor [AR], oestrogen receptor alpha [ESR1] and oestrogen receptor beta [ESR2] to the spatial distribution of grey matter differences and relates these findings to the development of sex differences in these brain regions. Finally these findings are specifically related to sex-specific associations in risk for Alzheimer's disease in polymorphisms of AR and ESR2.

3.2 General methodology

3.2.1 Recruitment

The morphological sex differences in the human brain were studied using 1013 structural T1 weighted MRI scans recorded in previously scanned healthy volunteers at the Wellcome

Trust Centre for Neuroimaging. These were independent from the set of those called back were re-screened for any previous neurological or psychiatric conditions. New T1-weighted 3D-MDEFT MRI images were recorded from each subject (n=260, 121 males, 139 females) and blood samples were taken for genotyping of polymorphisms in AR, ESR1, ESR2, PR and aromatase at the time of scanning. The genetic analyses were performed on Caucasians of European ancestry to avoid stratification artifacts. Subjects gave written informed consent and the local ethics committee approved the study.

3.2.2 Genotyping

Individuals were genotyped as discussed previously.

3.2.3 Imaging and image preprocessing

Imaging sequence for the T1-weighted 3D-MDEFT and image preprocessing were described in the methods chapter.

3.2.4 Imaging analysis

Brain volumes were extracted using the sum of the intensities from the native space tissue probability maps for grey matter, white matter and cerebrospinal fluid. In section one, these were used within a GLM on SPSS to determine whether sex differences in brain volume were associated with genotype. In section two, they were used as covariates of no interest after Gram-Schmidt orthogonalisation within the VBM analysis. VBM analysis was performed using 8 mm FWHM smoothed modulated grey matter probability maps looking for voxel-wise associations with genotype for AR(CAG)n, ESR1(TA)n, ESR2(CA)n, PROGINS, CYP19(TTTA)n and with 2D:4D. In section three, the VBM models were tested for a gene interaction with sex and age, as well as for a gene association with extracted hippocampal volume.

3.3 PROGINS polymorphism and whole brain volume

3.3.1 Introduction

Due in part to the ease of determining sex and size effects, sex differences in global brain measures have been extensively characterized. One of the most robust and well-replicated of these is in total brain volume with consistently greater brain volume in males than females. Studies consistently report about 10% greater brain volume in males than females across several age ranges from neonates to adolescents and adults even when controlling for factors such as body size (Goldstein et al., 2001b, Gur et al., 2002b, Gur et al., 1999a, Neufang et al., 2009, Gilmore et al., 2007). While even Broca has described the differences in brain size, several studies attempt to claim that this difference can be accounted for by factors such as surface area, height or weight and demonstrate that when correction using the ratio of brain size to body weight is used, this difference disappears. The problem with this approach has been explicated by Ankney, where he shows that this ratio decreases with increasing body size and using a covariance approach to perform the correction restores this difference. Body size, Ankney showed from autopsy data of 1261 adults, accounts for about 30% of the sex difference in brain volume. This is backed up by findings by Pakkenberg that men have about 23 billion neocortical neurons, while females have 19 billion(Rushton and Ankney, 2009).

In turn, females appear to have greater cortical complexity, a measure of gyrification and fissuration, within the association cortices (Luders et al., 2004). There is some evidence to suggest that women have a higher percentage of grey matter [GM], while men have a higher percentage of white matter [WM] and cerebrospinal fluid [CSF] although this finding is not as consistently replicated (Gur et al., 1999b). This finding could be explained by a general trend for larger mammalian brains to consist of proportionally more white matter (Zhang and Sejnowski, 2000). Regional volumes after correction for global

measures show similar consistencies across a range of techniques from VBM to manual volumetry and cortical thickness.

While these differences are present at birth (Gilmore et al., 2007), post-mortem data using brain weight suggest that this dimorphism continues to develop until the age of three years old, the period of greatest increase in brain volume, with no differences in slope between sexes beyond five years old and including puberty (Anatole and Doris, 1978). This suggests that these brain differences are not directly linked to body size as the sex differences in height and body weight show their greatest divergence during the pubertal years.

The specific mechanisms underlying gender differences in the brain remain poorly understood on the one hand because they are confounded by the complex influences of nature and nurture, and on the other because of the difficulty of experimentally isolating specific gender-related factors that may account for sex differences.In all of these dimorphic traits, there remain considerable overlaps between males and females. For example, around 60% of individuals are either male with brains smaller than the maximum brain size for females or vice versa. One may attribute this overlap either to the presence of factors unrelated to sex that influence these brain traits or by a continuum within each gender group of sexually dimorphic factors.

Heritability measures the fraction of phenotype variability that can be attributed to genetic variation and is divided into narrow sense heritability measuring at the proportion due to additive genetic variance and broad sense heritability measuring the proportion of variability due to all genetic contributions. Thus heritability can be considered to be synonymous with the genetic component of individual variability, although criticism has been levelled against the validity of heritability as an accurate measure of genetic variability. Several twin studies have demonstrated that individual variability in a number of volumetric brain measures has a large genetic component, with estimated heritability between 70 and 90% of total grey matter, white matter and whole brain volume in adults (Thompson et al., 2001, Peper et al., 2007, Goldman et al., 2008) (Bartley et al., 1997). Baare et al in a study of over 100 twin pairs found heritabilities of 90% of whole brain

volume, 82% of grey matter volume and 88% of white matter volume (Baare et al., 2001). Further links have been demonstrated between the heritable component of brain volume and of intelligence (Thompson et al., 2001, Cannon et al., 2006, Hulshoff Pol et al., 2006). These studies specifically examining heritable networks together with others that have delineated anatomical networks of regions that co-vary in volume provide a further means of querying the genetic basis for individual differences in brain structure.

Understanding the factors accounting for brain size may have both functional and medical significance. Indeed one of the hallmarks of autism, where the brain is theorised to have an 'extreme male pattern', is increased head circumference (Baron-Cohen et al., 2005). Brain volume correlates with intelligence, particularly performance IQ (Gur et al., 1999a, McDaniel). Additionally, functional capacity in Alzheimer's patients is related to premorbid brain volume over and above the rate of atrophy suggesting that brain volume provides reserve against cognitive decline (Mori et al., 1997). The ratio of total brain volume to intracranial or cerebrospinal fluid volume has also been found to be negatively associated with dispositional neuroticism (Knutson et al., 2001). Yet efforts to pinpoint causes for sexual dimorphism are confounded by the plethora of genetic and physiological differences between sexes. Despite the differences, there is considerable overlap in brain volume between sexes that can be exploited to circumvent these obstacles by considering the effect of genetic polymorphism on variation within sex. These polymorphisms have relatively discrete and consistent effects on the molecular level that can act from the beginning of development into the entirety of lifespan.

The progesterone receptor is an ideal candidate for this approach. The receptor is not only broadly expressed across the brain in both males and females, but also regulates neurogenesis and proliferation of neural progenitors (Brinton et al., 2008). The PROGINS allele of the human progesterone receptor gene, PR, is a 320 bp Alu insertion present in 10-15% of Caucasians. PROGINS diminishes response to progesterone in two ways. The insertion itself reduces efficiency of transcription of PR and decreases stability of its RNA transcript, but also increases oestrogen-induced transcription. The insertion is also in complete linkage disequilibrium with a V660L substitution that decreases transactivational

activity and opposes cell proliferation less efficiently when the receptor binds progesterone. This was shown by independent subcloning of individual polymorphisms for molecular genetics characterisation (Romano et al., 2007).

3.3.2 Methods

I investigated whether carrying the PROGINS allele was associated with differences in brain volume in 254 healthy volunteers. T1-weighted anatomical 3D-MDEFT MRIs were acquired for each and segmented into grey matter, white matter and cerebrospinal fluid using SPM5. The sum over the tissue probabilities in each class was used as an estimate of total brain compartment volume. Grey matter and white matter volumes were summed to calculate whole brain volume. I detected the presence or absence of the PROGINS allele by sizing and fluorescent detection of a PCR amplicon over the region of the insertion. I analysed for the effect of sex and genotype with a general linear model in SPSS accounting for age and scanner effects (*7*). Post-hoc analysis was performed for the other candidates, for whom an association with brain volume was not as strongly hypothesised and no significant association was found.

3.3.3 Results

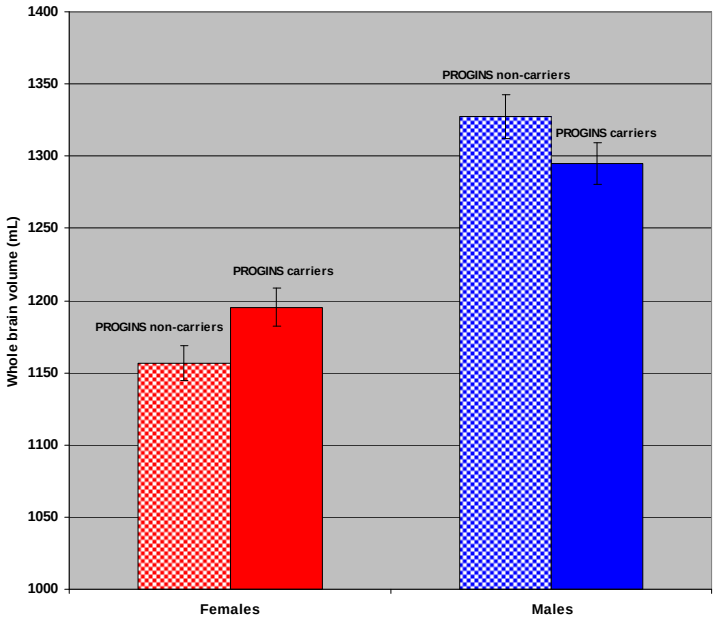


Figure 3.1 Comparison of whole brain volume between PROGINS carriers and non-carriers in males and females. Whole brain volume is shown in millilitres and grouped by sex and carrier status. Error bars reflect 95% confidence intervals.

The bar chart shows that brain volume is larger in female PROGINS carriers than female PROGINS non-carriers, but smaller in male PROGINS carriers than male PROGINS non-carriers. Females carrying the PROGINS allele have significantly larger brain volumes than females homozygous for the major allele (means of 1175 and 1120 mL respectively, p=0.011), while males carrying the PROGINS allele have significantly smaller brain volumes than males homozygous for the major allele (1248 and 1290 mL respectively, p=0.047). Within sex genotypic difference was about 30% of average sex difference. This relationship holds for grey (p=0.024) and white matter (p=0.011) volumes in females, but shows a non-significant trend in those of males. A significant cross-over interaction shows that the PROGINS allele has different effects in the two sexes (p=0.001). There is no significant interaction with age, suggesting that the effect is consistent across lifespan, and no influence of genotype on cerebrospinal fluid volume (p=0.697 in females, p=0.152 in males). Thus carrying the PROGINS allele lessens normal sex differences in brain volume.

3.3.4 Discussion

My findings demonstrate that the progesterone receptor has an early role in the development of sex-linked differences in brain volume, suggesting that in the progesterone-rich environment of the female brain, it opposes brain growth. Conversely, the receptor may have a trophic effect in the male brain. Indeed progesterone appears to down-regulate androgen receptor expression and influences oestrogen signalling, so might act differentially via those pathways (Brinton et al., 2008). Progestins are prescribed during pregnancy and prenatal exposure has been associated with increased IQ in girls, greater early academic achievement and earlier walking, for which an effect on brain volume may provide a potential mechanism (Wagner, 2006, Gur et al., 1999a).

The discovery that a common polymorphism accounts in part for basic sex difference in brain volume offers further promise for investigating broad biological influences on human brain variation using relevant candidate genes.

3.4 Sex differences in regional brain volume

3.4.1 Introduction

As described earlier, the regional differences in brain volume between males and females are well-established. Males appear to have greater relative grey matter volume in the amygdala, hypothalamus, frontomedial cortex (Goldstein et al., 2001a), hippocampi, medial temporal lobes and anterior cerebellum (Good et al., 2001). Females appear to have relatively more grey matter in the inferior frontal, lateral orbital and middle temporal cortices, the planum temporale and inferior parietal and cingulate gyri, after correction for brain size (Luders et al., 2006, Luders et al., 2003, Good et al., 2001). While the sexual dimorphism of these regions have been extensively described, it remains unclear how much

these volumetric differences relate to known differences in personality and cognition or which regions might be influenced by one biological factor or another.

In a recent VBM study by Witte et al of 34 young adults, 17 males and 17 females, grey matter volume of the sexually dimorphic left inferior frontal gyrus was found to correlate with circulating levels of estradiol and to correlate inversely with levels of testosterone. Progesterone levels were related to the volume of the right temporal pole. These observations might be interpreted to reflect structural differences resulting from more recent variation in hormone levels between individuals, but it is unclear whether they reflected organizational influences during puberty or earlier because of individuals with currently high hormone levels also having higher levels during those critical periods. Significant variance was probably also introduced by circadian and monthly variation although the authors made an effort to take female hormone levels in the follicular phase of their menstrual cycle. Additionally testosterone is able to act on both the oestrogen and androgen receptors, so it would be difficult to determine the specific receptor pathways through which these hormones might be acting (Witte et al., 2009).

A more direct means to investigate the effect of hormones on the brain makes use of individual variation in the expression of the androgen (AR) and oestrogen (ER) receptors. The primary effects of testosterone occur through the activation of the androgen receptor, a nuclear transcription factor. Longer repeats of the polymorphic polyglutamine tract in the N-terminal exon of AR inhibits its interaction with co-activators and the transcription of its gene to mRNA (Choong, 1996, Chamberlain et al., 1994, Beilin et al., 2000). AR CAG repeat length has been negatively associated with performance on the mini-mental state exam, digit symbol coding and Trails B, tests of general cognition and processing speed (Yaffe et al., 2003). It has also been positively correlated with some aspects of neuroticism and femininity (Jönsson et al., 2001, Loehlin et al., 1999) and negatively with psychoticism (Turakulov et al., 2004). Whereas one previous structural imaging study suggested an influence of the AR CAG repeat polymorphism on changes in white matter volume during adolescence (Perrin et al., 2008), there are no data about the relative effects of these polymorphisms on different brain regions in the two sexes at different ages.

In this section I investigate the extent to which sexually dimorphic regions are influenced by polymorphisms of AR CAG, ESR1 TA and ESR2 CA and CYP19 TTTA repeat length on regional grey matter differences by voxel-based morphometry.

3.4.2 Methods

Recruitment, genotyping, imaging and VBM analysis was performed as described in the methods chapter.

Age range	5-15	15-25	25-35	35-45	45-55	55-65	65-85	Total
Number	33	333	333	150	76	62	26	1013

Gender	Male	Female	Total
Number	615	398	1013

Handedness	L-handed, L-legged	L-handed, R-legged	R-handed, L-legged	R-handed, R-legged	Total
Number	15	20	21	957	1013

Ethnicity	White	Black Caribbean	East Asian	South Asian	Latin American	Other/Mixed	Total
Number	955	22	17	13	6	11	1013

Table 3. 1 Tables showing demographic distribution of people used in the sex and sex by age interaction analysis. Subjects were all scanned on the Sonata 1.5 T scanner on the 3D-MDEFT sequence and completed a standard screen to exclude any neurological or psychiatric conditions.

I first performed VBM analysis in a sample of 1013 individuals independent from the main genetics database to determine regions of the brain that were significantly different between males and females. Regions from the comparison were subsequently compared with regions shown in subsequent analysis by genotype. VBM analysis was performed with each polymorphism independently for AR(CAG)n, ESR1(TA)n, ESR2(CA)n, CYP19(TTTA)n and PROGINS. As described in the methods, a split was used for microsatellite polymorphisms to divide alleles into high and low repeats and this was based on the literature or with a median split. Thus genotype was defined as a three level condition, grouping by short homozygotes, heterozygotes and long homozygotes. Additional analysis not shown provided a better model using a continuous variable averaging the two allele repeat lengths. Sex was used as an independent condition, while age and GM volume were used as covariates after Gram-Schmidt orthogonalisation. F-tests were first performed in order to determine regional associations, then individual T-tests were performed in order to determine the direction of the effect and to test for interactions.

All regions reported in the results were significant after family-wise error correction on the whole brain level unless stated otherwise. Correction for comparisons between gene polymorphisms was not performed as there were independent *a priori* hypotheses for each. Figures were displayed using a threshold of $p<0.001$ uncorrected, however thresholds for figures were sometimes adjusted for display purposes and stated in figure captions as some associations were highly significant.

3.4.3 Results

3.4.3.1 Sex differences

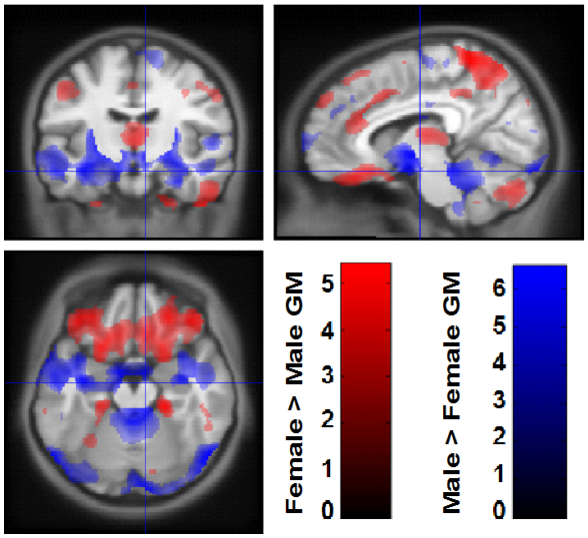


Figure 3.2 Regions showing significant sex differences in GM volume. Red shows regions with greater GM volume in females. Blue shows regions with greater GM volume in males. T-statistic maps thresholded at p>0.05 fwe-corrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

I first delineated sex differences in brain structure within my cohort. Modulated, normalised grey matter maps, derived from structural magnetic resonance scans, were smoothed and compared by VBM. Females show relatively greater GM volume than males around the central sulcus and superior temporal and ventrolateral prefrontal cortices. Much of the increased grey matter volume in males compared to females is situated inferiorly around the temporal lobes and cerebellar hemispheres.

3.4.3.2 Regional grey matter volume correlated with AR CAG repeat length

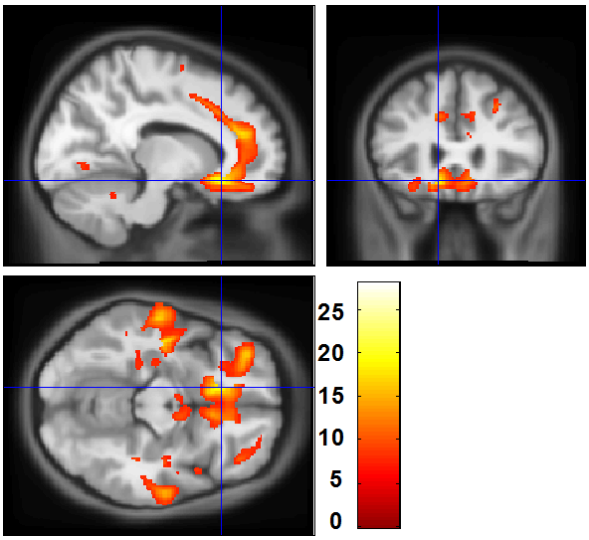


Figure 3.3 Regions significantly associated with AR(CAG)n in GM volume. F-statistic maps thresholded at p>0.05 fwe-corrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

I next sought to determine regions in which grey matter volume is correlated with AR CAG repeat length. Grey matter volume was positively associated with the longer AR(CAG)n within the anterior cingulate cortex, orbitofrontal cortex, temporal lobes, and hypothalamus. In the superior temporal gyrus and insula bilaterally, grey matter increased with increasing CAG repeats significantly more in males than females. Within the temporal lobe there were a number of distinct clusters in each of which grey matter volume was positively correlated with CAG repeat number in both males and females. Other positively correlated areas were found in the right supra-genual cingulate cortex, right inferior cerebellum, left dorsomedial prefrontal and right inferior parietal cortex. A homotypic cluster in the left inferior cerebellum was also significantly positively correlated.

3.4.3.3 Regional grey matter volume correlated with ESR1-alpha TA repeat length

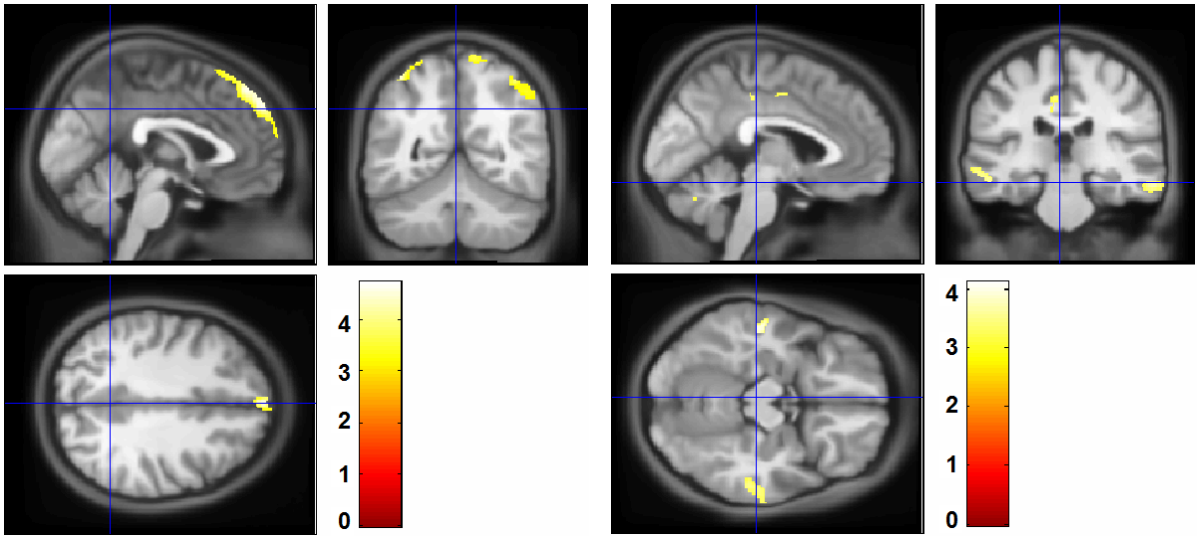


Figure 3.4 Regions significantly associated with ESR1(TA)n in grey matter volume. Figure on the left shows regions that were positively associated with ESR1(TA)n and the figure on the right shows regions that were negatively associated with ESR1(TA)n. T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

In both males and females, grey matter volume correlated negatively with number of TA repeats in both temporal lobes, in the same region as that showing a correlation of grey matter volume with increasing AR CAG repeats. With a prior hypothesis and the known sex differences in cingulate gyrus, I tested this structure specifically with small volume correction using the cingulated gyrus as an ROI and found that left dorsal middle cingulate cortex also showed a significant correlation with ESR1-alpha polymorphism. This same structure also showed significantly greater grey matter volume in females than males.

3.4.3.4 Regional grey matter volume correlated with ESR2(CA)n

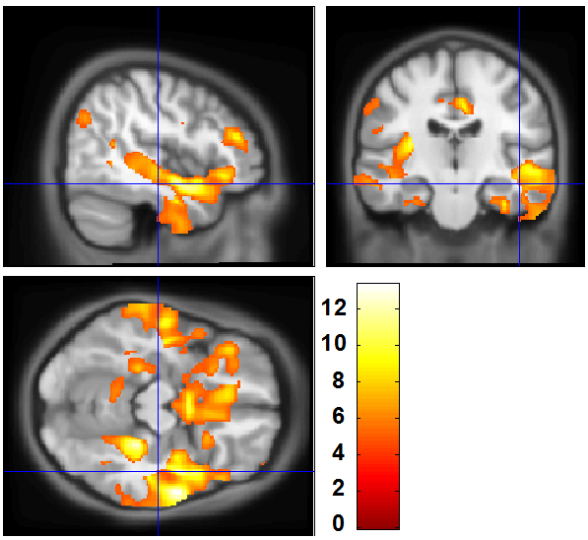


Figure 3.5 Comparison of regions with significant association with ESR2(CA)n in GM volume. F-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

Similar regions were associated with ESR2 genotype as with AR CAG repeat length in particular in the temporal lobes involving the parahippocampal and superior temporal gyri, hypothalamus, orbitofrontal cortex, insula, anterior cingulate and lateral prefrontal cortex. This was found to be primarily driven by an association in women and not in men and GM volume was negatively associated with ESR (CA)n.

3.4.3.4 Regions associated with sex hormone polymorphisms and digit ratio

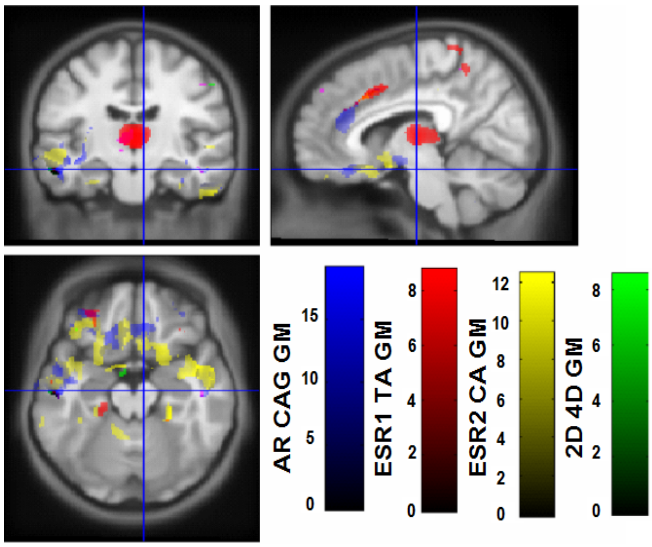


Figure 3.6 Regions associated with AR(CAG)n, ESR1(TA)n, ESR2(CA)n and 2D:4D. F-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

Broadly AR CAG repeats and ESR2 CA repeats were associated with similar regions within the temporal lobes, hypothalamus and orbitofrontal cortex. The left lateral orbital gyrus and left temporal cortex appeared to be associated in volume with digit ratio and AR, ESR1 and ESR2 polymorphism. Using masks of sex difference effects to constrain analysis of genotype associated structural differences, I found that both inferior temporal lobes are significantly larger in males than in females, and that their GM volume correlates positively with AR CAG repeat length. These regions also coincided with regions found to be sexually dimorphic. No regions were found to be directly associated across sex with the gene for aromatase, CYP1A1, or the progesterone receptor, PROGINS.

3.4.3.4 Conjunction analysis of AR(CAG)n and ESR2(CA)n in females

I then performed formal conjunction analysis with the more conservative conjunction null asking the question: Which regions are significantly associated with both AR(CAG)n in both sexes and with ESR2(CA)n in females? Thus clusters surviving significance jointly refute both null hypotheses with false positive rate controlled over all null outcomes (Friston et al., 1999, Friston et al., 2005).

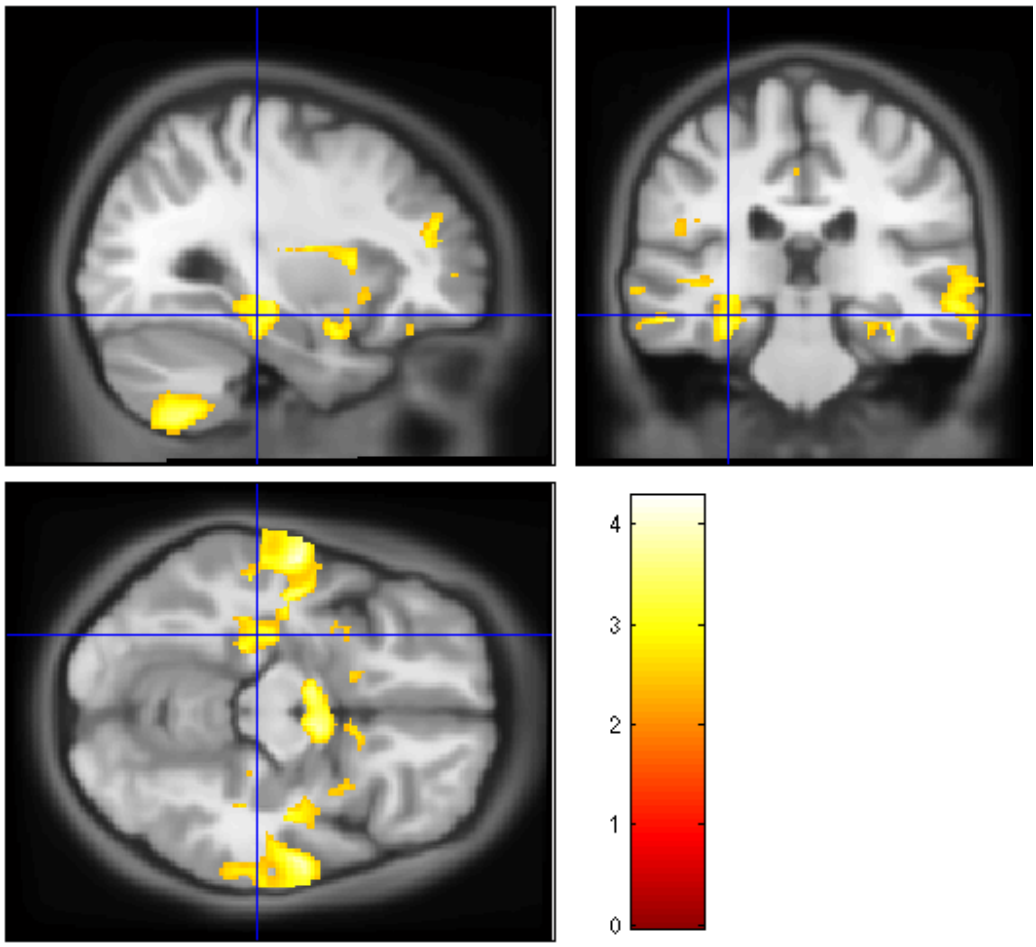


Figure 3.7 Conjunction analysis showing regions associated with both AR(CAG)n and ESR(CA)n in females.

Clusters surviving $p<0.05$ family-wise error correction included the middle temporal gyrus, hippocampus, cerebellar tonsil and hypothalamus bilaterally.

3.4.4 Discussion

The brain regions I have implicated as sexually dimorphic are in broad agreement with the literature (Neufang et al., 2008, Sowell et al., 2007, Arnold, 2004, Raz et al., 2004, Gur et al., 2002a, Good et al., 2001). The relatively greater grey matter volume in the cerebellum, inferior temporal lobes and inferior frontal lobes in males and around the central sulcus and ventrolateral prefrontal cortex in females are similar to patterns found using techniques such as voxel-based morphometry, manual volumetry and cortical thickness measurement. Most of the areas of greatest correlation with AR CAG repeat length were also different between the sexes. However these regions, which consistently showed a positive correlation with greater repeat length, were not always larger in one sex than the other. While the inferior temporal cortex was larger in males, the anterior cingulate cortex was larger in females. Moreover, the loci of greatest sex difference were proximal but not coincident with the loci of greatest correlation with AR CAG genotype. The same applied to a region in the middle temporal gyrus bilaterally, which demonstrated a marginally significant interaction between sex and genotype, but was located between the loci of the two main effects (of sex and AR CAG status). A possible explanation may be methodological in that hormonal sex differences may be detectable because of differing spatial extents of hormonal influence, rather than differing sizes of effect.

Longitudinal studies suggest that there is great variation in individual age trajectory (Raz et al., 2004); thus it is likely that subtle sex differences in brain ageing may require the sensitivity provided by my cohort of above 1000 individuals which provides a power greater than in other studies to date. Similar sex differences in developmental and age trajectories have been demonstrated in subcortical structures such as the putamen and thalamus (Gur et al., 2002b, Raz et al., 2004, Xu et al., 2000, Neufang et al., 2008) and relative preservation of the cerebellum in females compared to males (Oguro et al., 1998, Sullivan et al., 2004) in studies that have mainly involved univariate measures derived from manual volumetry. The finding of relatively greater age-related bilateral posterior insula grey matter reduction in females, a region commonly excluded in manual volumetric studies, is novel.

I find strong evidence for a differential and selective effect of genetic polymorphisms of both the androgen and oestrogen receptors on human brain structure in a healthy adult population. This result complements current knowledge of the influence of sex hormones on brain development and ageing. For example, recently it was shown that pubertal hormones result in neurogenesis in sexually dimorphic regions (Ahmed et al., 2008). My results show that individuals with a longer polyglutamine segment in exon 1 of the AR have relatively more grey matter in the temporal lobes, orbitofrontal cortices, inferior cerebellar hemispheres, right anterior cingulate, right insula and right inferior parietal cortices than those in whom the segment is short.

There is additional support from pharmacological neuroimaging studies for a specific involvement of sex hormones in the determination of the morphology of the brain structures I identify. Testosterone normalises PET activity induced by sexual stimuli in the orbital and inferior frontal cortices, claustrum and insula in hypogonadal men as well as parietal activity during mental rotation (Redoute et al., 2000). Increased testosterone correlates positively with superior frontal and negatively with orbitofrontal cortex activity, thought to reflect a modulation of testosterone-induced changes in amygdala regulation (van Wingen et al., 2008). Similar findings have been found in response to social threat, with greater activation to angry versus happy faces in orbitofrontal cortex with testosterone administration (Hermans et al., 2008). Testosterone has also been shown to increase cerebral metabolism in the posterior and sub-genual anterior cingulate and right parietal cortices in women with anorexia nervosa (Miller et al., 2004). Of note, relative amygdala volume did not correlate with AR CAG repeat length. This may be because it is regulated by a number of the implicated cortical regions whose activities are themselves directly modulated by testosterone. Thus, the regions I identify, which are broadly implicated in social and emotional functions, show positive correlations between local grey matter volume and AR CAG repeat number. Testosterone levels also modulate local activity in these regions.

The greatest effect of AR CAG and ESR2 CA length is found in the temporal lobes bilaterally, stronger on the left. The temporal lobes appear to be a key area associated with sexual dimorphism and a number of studies provide strong support for prominent sex hormone influences on this part of the brain (Janowsky, 2006). Some reports suggest faster age-related temporal lobe atrophy in men (Cowell et al., 1994), with higher local cortical glucose metabolism in men than women (Gur et al., 1995). There are similar sex differences in BOLD activation during passive listening (Phillips et al., 2001). Measurement of post-mortem concentrations of female brain testosterone levels reveal that the highest brain concentrations after hypothalamus and substantia nigra are found in the temporal cortex (Bixo et al., 1995). Enzymes necessary for the synthesis of androgen, such as 5-alpha reductase, are also highly expressed within the temporal cortex (Stoffel-Wagner et al., 1998).

3.5 Alzheimer's endophenotype

3.5.1 Introduction

The association of the temporal lobe with genetic variation of the sex hormone polymorphisms is of significance for a number of reasons. The temporal lobes particularly the hippocampus and regions within the medial temporal lobe undergo early and relatively selective atrophy during Alzheimer's disease. Women are more likely to develop Alzheimer's disease than are men and have been found to have an earlier age of onset in some studies. In men, possessing a short allele of the AR CAG repeat polymorphism has been shown to confer a 2.5 fold risk of Alzheimer's disease and a 15.3 fold risk of early onset Alzheimer's disease [less than 65 years of age] (Lehmann et al., 2003). The same group subsequently found that this risk attenuated by higher serum testosterone (Lehmann et al., 2004). In women, ESR2 genotype is associated with a 1.5-2 fold increase in risk for Alzheimer's disease (Pirskanen et al., 2005). AR and ESR2 are both Alzheimer's risk genes. The aim of this section was therefore to investigate endophenotypes for Alzheimer's that could be influenced by these risk genes in order to contribute to an understanding of

mechanisms by which they confer risk. The temporal lobe clusters in the whole brain VBM analysis have not been validated and accepted in the literature in the same way premorbid hippocampal volume and atrophy rates have as an early biomarkers for memory in healthy individuals (Van Petten, 2004), apoE4-related disease risk (Mueller et al., 2008, Lema tre et al., 2005, Tohgi et al., 1997), subsequent cognitive decline and the development of or pathological confirmation of Alzheimer's disease (Schuff et al., 2009, Jack Jr, 1997, Gosche et al., 2002). The method of automated hippocampal volumetry using DARTEL with a hippocampal atlas has been shown to be accurate with high overlap with the voxels identified by manual volumetry (Yassa and Stark, 2009b). The investigation of these markers in adults across lifespan may be particularly relevant here because of the increased risk of AR(CAG)n in the younger below 65 year cohort. Additionally as can be seen from the demographics provided, the number investigated in the above 45 years age range is comparable to or larger than many studies investigating hippocampal volume with ApoE4 genotype. It is also interesting to note that in the subset of 18 participants undergoing verbal memory testing, 24 hour recall of newly learned foreign words was significantly associated with AR(CAG)n. One might therefore expect that these polymorphisms, now found to modulate temporal lobe volume would do so in a way congruent with Alzheimer's risk between sexes, with AR(CAG)n showing a male-selective effect and ESR2 (TA)n showing a female selective effect within the temporal lobe if I were to look for regions with a sex by gene interaction. I would also expect the temporal lobes to demonstrate these sex interactions more significantly than in other parts of the brain.

Indeed, testosterone and oestrogen have been shown to be important modulators of memory in old age. Testosterone supplementation in hypogonadal men improves verbal memory. However this may be caused either by its direct action on androgen receptors or by aromatisation of testosterone into oestrogen, that acts on oestrogen receptors. Administration of the non-aromatisable androgen dihydrotestosterone, on the other hand, improves spatial memory and not verbal memory. Thus brain androgen influences spatial memory, while aromatised brain oestrogen influences verbal memory. Studies of the effect of exogenous or endogenous testosterone levels on cognition have been made recently in diseases involving androgen imbalance at various stages of life. Females with congenital adrenal hyperplasia, with *in utero* overproduction of androgens, have similar performance on tests of spatial cognition as males (Puts et al., 2008), while children with higher

amniotic testosterone levels exhibit more autistic traits (Auyeung et al.). Thus, it would appear that spatial cognition and the preponderance of autism in males can be attributed in part to the effects of androgens in early development, while serum free testosterone levels predict memory and visuo-spatial abilities in elderly men (Moffat et al., 2002).

Studies demonstrate that larger initial hippocampal volume is associated with reduced risk and later onset of Alzheimer's perhaps because increased size provides greater 'cognitive reserve'. In 511 cognitively normal elderly individuals who were free of dementia, the 35 who subsequently developed dementia had smaller hippocampal and amygdalar volumes (den Heijer 2006). Hippocampal volume also appears to be a good marker for genetic risk. For example, APOE4 gene carriers have been found to have reduced right hippocampal volume, particularly after the age of 65 and a subsequent study found that homozygotes have medial temporal lobe atrophy including the hippocampi as compared to heterozygotes and noncarriers of APOE4 (Lemaitre, 2005). Additionally hippocampal atrophy has been shown to be independently associated with the risk of Alzheimer's disease regardless of APOE4 genotype suggesting that other factors in addition to APOE4 are important in this process. In a recent study comparing AD patients, MCI patients and controls, hippocampal measures were found to predict subsequent deterioration independently of whole brain atrophy rates. In patients with MCI specifically, hippocampal baseline volume and atrophy rate predicted progression to AD. (Henneman 2009). I thus tested for a specific endophenotype, hippocampal volume and its atrophy with age, both factors that appear to be related to risk for Alzheimer's disease.

3.5.2 Methods

Recruitment, genotyping, imaging and initial analysis was performed as in the last section.

I first analysed for a sex by genotype interaction between regional GM volume and ESR2(CA)n and AR (CAG) by VBM, with the prior hypothesis that regions of significant association with genotype or sex would be more likely to exhibit an interaction. Contrast estimates were displayed in order to view the respective genotype effect in each sex Due to the regional hypothesis based on Alzheimer's disease associations and temporal lobe

association, hippocampal volume was extracted from the DARTEL-warped modulated grey matter density maps using AAI ROIs with the Wake Forest University Pickatlas on SPM(Maldjian et al., 2003). This methodology for volumetric extraction has been validated against other cross-participant registration techniques and manual morphometry(Yassa and Stark, 2009a). Individual hippocampal volumes were used as variables in a bivariate correlation with genotype on SPSS. Finally the regions were tested for an age by genotype interaction in order to determine whether age-related differenes were also modulated by genotype. Thus two structural endophenotypes for Alzheimer's disease were tested for a gene association in order to determine if they could be the neural substrates underlying the gene associations with disease.

3.5.3 Results

3.5.3.1 Regions showing interactions between sex and genotype

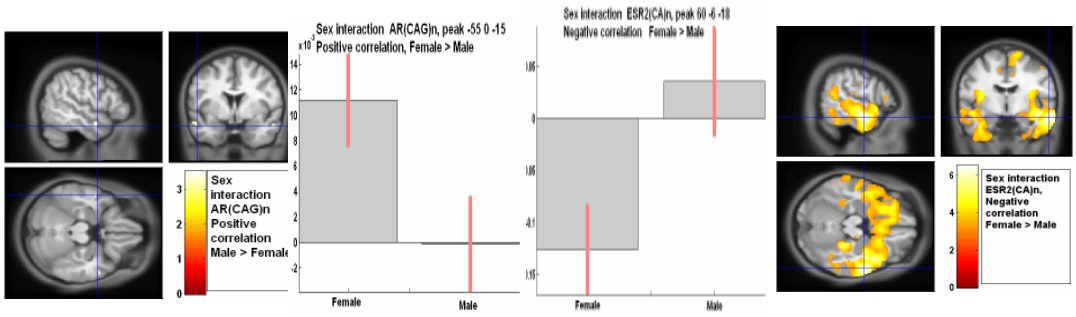


Figure 3.8 Sex by genotype interaction in GM volume of the temporal lobes with AR(CAG)n and ESR2(CA)n. The left shows the sex interaction with AR(CAG)n and a plot of its contrasts. The right shows the sex interaction with ESR2(CA)n and a plot of its contrasts. T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted images warped together into MNI space. The peak voxel for the sex interaction was extracted to plot contrasts.

Having determined regions in which grey matter volume is significantly associated with the androgen and oestrogen receptor polymorphisms, I investigated the relationship to sex chromosome associated differences in brain structure first by defining any overlap with regions showing sex chromosome-based differences and secondly, by examining for an interaction between brain volume changes associated with AR CAG length-dependant genotype and sex.

I hypothesized that regions affected by AR genotype would overlap with regions that differed between the sexes because males have higher circulating levels of testosterone and so might show greater effects of AR CAG repeat length than females. Small volume correction using a 12mm sphere centered on the most significant voxel in an area showing greatest correlation of GM volume with genotype revealed a proximal region in the middle temporal gyrus that demonstrates a significantly greater increase in GM volume with longer

CAG repeat length in females than males. This interaction of chromosomally defined sex with AR genotype was present bilaterally, but was confined to a small part of the temporal cortex. Overall, it was apparent that temporal lobe volume is increased in individuals with greater CAG repeat length in both males and females.

On the other hand, the sex interaction with ESR2 CA repeat length was much more significant and survived global correction. Females with longer repeats and consequently decreased expression of ESR2 appeared to have smaller temporal lobe volume than those with shorter ESR2 CA repeats. In males on the other hand, there was no such association and there appeared to be a trend in the reverse direction.

3.5.3.2 Hippocampal volume and the association with AR(CAG)n and ESR2(CA)n genotype

		Bilateral hippocampal volume	
		Correlation coefficient	P-value
AR(CAG)n	F	0.16	0.048
	M	0.0815	0.36
	F+M	0.1435	0.018*
ESR2(CA)n	F	-0.22	0.008*
	M	-0.024	0.7898
	F+M	-0.11	0.082

Table 3. 2 Correlation of hippocampal volume with AR(CAG)n and ESR2(CA)n in males and females. Bivariate correlation was performed between genotype and hippocampal volume for each sex and across sex and uncorrected p-values displayed. Results surviving Bonferroni correction for multiple comparisons are marked with an *.

Bilateral hippocampal volume was associated with both ESR2 and AR genotype in females. With the AR gene, females showed a positive correlation with CAG repeat length, while there was a weak trend towards a positive correlation in males. When combined across sex, hippocampal volume was overall significantly positively correlated with CAG repeat length. With the ESR2 gene, females showed a strongly negative correlation with CA repeat length, while there was no effect at all in males and no significant association when males and females were combined.

3.5.3.3 Interaction AR(CAG)n with age-related atrophy

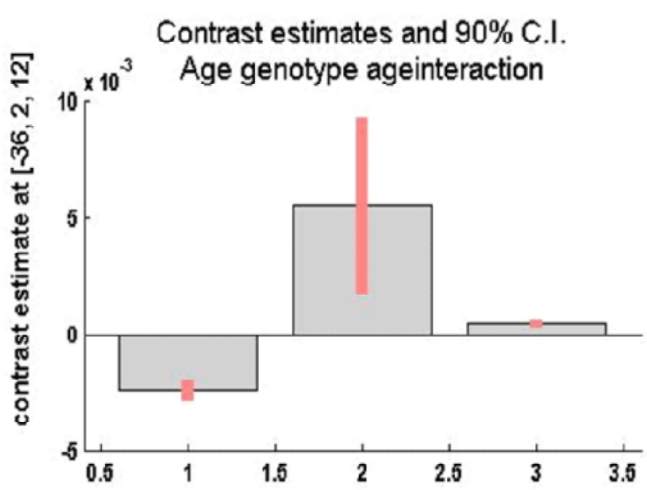


Figure 3.9 Plot of contrast estimates of age, genotype and age by genotype interaction in the temporal lobe. Peak voxel from an analysis of age interaction after small volume correction of an 8mm sphere around the peak voxel from the main effect of genotype was extracted to look at effects of age, genotype and the interaction between the two.

Due to the known influences of hormone replacement therapy on cognitive ageing (Janowsky, 2006), I then explored whether the androgen receptor CAG polymorphism modulates grey matter volume changes associated with normal brain ageing. Males showed significantly slower grey matter atrophy in the posterior insula bilaterally than do females. On the other hand, females appeared to show relative preservation of grey matter volume in the right anterior putamen, right anterior thalamus and left superior cerebellum with increasing age.

To test this idea I performed a conjunction analysis (Friston et al., 1999)of regions in which grey matter volume is significantly correlated with both age and AR CAG genotype. I thus determined whether there are brain regions with a significant interaction between genotype and age, before analysing for a three-way interaction between age, sex and genotype (threshold, $p<0.05$ fwe-corrected for whole brain). In the conjunction analysis, temporal lobe volume was significantly correlated with both age and AR CAG repeats. Normal age-related atrophy was significantly slower with increasing CAG repeat length in the left superior insula (also detected as a trend on the right at $p<0.001$ uncorrected) and left

dorsolateral prefrontal cortex. In an analysis of the orbitofrontal cortex suggested by its significant correlation with CAG AR genotype a similar interaction was found (using a statistical threshold based on a small volume correction centred on that cortex). There was no significant three-way interaction between sex, age and AR genotype.

There was no significant interaction between ESR2 genotype and age-associated hippocampal or temporal lobe volume differences.

3.5.4 Discussion

Both AR CAG and ESR2 CA repeat length are associated with hippocampal and temporal lobe volume in women. AR CAG also appeared to influence temporal but not hippocampal volume in men, while ESR2 showed a highly significant association in both the hippocampi and temporal lobes of women. While the female specific association with temporal and hippocampal volume is completely consonant with previous genetic studies, the female specific association with AR CAG repeats runs counter to the original hypothesis. There are a number of possible explanations that can reconcile the apparent disparity between this finding and those of previous genetic association studies. Firstly, no association was sought with AR CAG and risk for Alzheimer's in women in previous studies as the polymorphisms was only genotyped in men. Thus it is possible that the AR CAG polymorphism does confer risk for AD in women, but that has yet to be tested. Additionally, it has been shown that risk in men was modulated by testosterone levels, where the short repeat allele conferred greatest risk in men with low testosterone levels. As I am looking at a relatively subtle endophenotype, it might be that the trend observed with hippocampal volume could yield significance with greater numbers and that there was additional variance due to the wider range in testosterone levels amongst men.

In the analysis of age-related atrophy, how appropriate is the use of cross-sectional age effects? In a large representative population the difference in a trait between individuals of different ages is due primarily to age-related longitudinal change as all other non-confounded effects would not be different between age. The main confounder would be cohort effects such as inter-generational nutritional and socioeconomic differences. Now

supposing the worst case scenario where cohort effects were significant in this dataset and cross-sectional age-related differences gave distorted estimates of longitudinal measures of age, how would the results of a gene by age interaction change as compared to an accurate marker or direct comparison with longitudinal age atrophy? Cohort effects are understood to be purely environmental and there is no reason why after equating ethnic and geographical background a group of individuals with one form of a gene should experience the cohort effect and another group should not. Thus cohort and other inter-individual effects are likely to affect both groups equally. Longitudinal studies have the advantage of overcoming the noise from inter-individual differences in the estimation of change and are therefore more sensitive, however they suffer from their own systemic errors such as scanner drift.

Loci of greatest correlation between relative grey matter volume and AR CAG repeat number coincide with those where age-related atrophy is greatest, a finding confirmed statistically in the temporal and orbitofrontal cortices. This effect may thus be partly due to hormonal influences on normal ageing rather than on early fetal or childhood development. Longer AR CAG repeat length was associated with slower age-related atrophy in the left insula and left dorsolateral prefrontal cortex as well as orbitofrontal cortex, with a trend also observed in the right insula. This finding suggests that hormones may mediate the observed sex differences in age trajectory within the insula, but not within subcortical and cerebellar structures, a hypothesis supported by the study of Neufang *et al* in children (Neufang et al., 2008).

At the molecular level, what could be the significance of a positive correlation between longer AR CAG repeats and grey matter volume, particularly in the temporal lobes? A longer CAG repeat expansion results in lower androgen receptor expression (Choong, 1996), and decreased activation of transcription by a subset of its co-activators (Beilin et al., 2000, Chamberlain et al., 1994). As previously mentioned, left temporal lobe grey matter density is significantly reduced in Klinefelter syndrome where there is over-expression of the AR due to increased gene dosage; this effect is reversed with testosterone supplementation (Patwardhan et al., 2000). Female sex, where testosterone levels are low but androgen receptor expression significant, is consistently associated with higher

Alzheimer's disease risk (Lindsay et al., 2002, Launer et al., 1999). A comparison of risk of Alzheimer's disease between men with long and short forms of the AR CAG polymorphism, that took into account serum testosterone levels, found increased risk with short androgen receptor repeats and lower serum testosterone levels (Lehmann et al., 2004). Temporal lobe volume is also partly predictive of AD risk (Kloppel et al., 2008, Jack Jr, 1997), Taken together, the evidence suggests that androgen receptor occupancy relates to temporal lobe grey matter volume. This effect could be mediated by a number of mechanisms, from a specific detrimental effect of unoccupied androgen receptors, to the relative influence of the androgen and oestrogen receptors on those regions. Indeed, as mentioned, oestrogen, in the form of hormone replacement therapy, has beneficial effects particularly on memory (Sherwin, 2002), at least in part a temporal lobe function. Furthermore, aromatase, the enzyme that converts testosterone into oestrogen, is highly expressed within the human temporal lobe (Steckelbroeck et al., 1999). Conceivably, higher androgen receptor expression could decrease the unbound pool of testosterone available for cross-binding and trans-activation of the oestrogen receptor (Lund et al., 2006) or conversion to oestrogen, which has neurotrophic and neuroprotective effects (Granholm, 2000, Behl and Holsboer, 1999).

3.6 Summary

I describe the influences of polymorphism of sex hormone-related genes across a number of measures of global and regional brain volume. I discover that the PROGINS mutation, associated with decreases in both expression and activity of the progesterone receptor, diminishes sex differences in overall brain volume as well as grey and white matter compartmental volume, but not cerebrospinal fluid volume. Regional associations with sex hormone-related gene polymorphisms were found in the temporal lobes, hypothalamus and orbitofrontal cortex. On further investigation of the temporal lobe influences by AR(CAG)n and ESR2(CA)n, I found that two endophenotypes of Alzheimer's were associated- ESR2(CA)n was associated with hippocampal volume in females and AR(CAG)n was associated with age-related atrophy of the temporal lobes.

3.7 Relevant conference presentations and manuscripts

Geoffrey CY Tan, Christopher WG Ho, Ese E Mudanohwo, Chia-Yeh C Chu, John Ashburner, , Nicholas W Wood, Richard SJ Frackowiak. Multiple influences of the androgen receptor polyglutamine polymorphism on the healthy human brain. Organisation for human brain mapping 2008, Melbourne Australia. Poster 288.

Geoffrey CY Tan, Christopher WG Ho, Carlton Chu, Nicholas Wright, Ese Mudanowho, Rachael Maddock, Jon Roiser, John Ashburner, Nick W Wood, Richard SJ Frackowiak. Multiple influences of sex steroid hormone receptor microsatellite polymorphisms on the healthy human brain. (Manuscript under review)

Geoffrey CY Tan, John Ashburner, Nick W Wood, Richard SJ Frackowiak. Progesterone receptor polymorphism modulates sex differences in human brain volume. (Manuscript under review)

CHAPTER 4

BRAIN CIRCUITS: SEX DIFFERENCES IN STRUCTURAL COVARIANCE, WHITE MATTER VOLUME AND FRACTIONAL ANISOTROPY

4.1 Abstract

White matter contains the axons comprising the long-range connections between cortical and subcortical neurons. Thus where grey matter differences exist in volume or composition, one might expect to find differences in white matter volume proximally and in the structural connectivity of those regions. Information about concomitant white matter changes can in turn elucidate the circuits and anatomical networks underlying changes observed regionally. This chapter thus considers these circuits and networks in terms of 3 approaches. The first section considers how anatomical covariance or the common variance between regions is influenced by polymorphisms of genes in the sex hormone pathways. The second section considers the influences of these polymorphisms on white matter volume and integrity. The third results section investigates how these circuits are influenced by a polymorphism that has been found to confer sex-specific risk for autism. The analysis for the first section was performed jointly with Dr Christian Lambert and sent as an abstract to the Organisation for human brain mapping 2009 where it was presented as a poster. The third was written as a paper with Mr Thomas Doke, who performed part of the analysis for his BSc (intercal) dissertation. Overall the chapter presents a dissection of molecular factors underlying the sexual dimorphism in overall neural circuitry in the realms of anatomical covariance and white matter, as well as an example of how this approach may be similarly applied to the investigation of sex-specific disease risk.

4.2 Gender asymmetries in amygdala emotive networks

4.2.1 Introduction

A number of studies have now demonstrated that certain structures of the brain appear to vary in a coordinated way across individuals. Thus when 1 region is relatively larger in an individual, one can expect certain other regions to be similarly larger. These covariances have been attributed to common trophic influences, structural connectivity and common environmental influences. Thus one can infer common anatomical networks or modes based on the covariance structure of regional volume across the brain. However patterns of cortical covariance appear to be relatively consistent across populations with few differences between males and females(Mechelli et al., 2005).

The major exception to this rule is the structural covariance of the amygdala, which is both sexually dimorphic and modulated by sexual orientation. Mechelli et al found the left amygdala to be positively associated with the left and right anterior inferior temporal cortex in males and with the right angular gyrus in females. The amygdala is thought to be active during tasks involving encoding and long-term memory for emotional stimuli as well in direct response to a range of emotional stimuli. A number of studies demonstrate sex-related lateralisation of amygdala response even in the absence of behavioural differences. For example Cahil et al (2001) describe an association of right amygdala activity to accuracy of recall in men, but left amygdala activity in women. This was further investigated by Canli et al (2002), who demonstrated that this accuracy-activity association was restricted to the most emotionally arousing stimuli. More recently, this difference has been also demonstrated in resting state functional connectivity (Kilpatrick 2006). Activity in BA21 and BA38 of the temporal cortex, the prefrontal cortex, BA17/BA18 of the occipital cortex, the insula, pulvina, the caudate and putamen was shown to be more strongly positively correlated with right amygdala activity in men. In women, left amygdala activity was more positively correlated with similar regions of the temporal, prefrontal and occipital cortex as well as the subgenual anterior cingulate, hypothalamus

and ventral anterior nucleus of the thalamus. While recent studies have suggested that individual differences in functional activity have a structural basis in white matter connectivity and grey matter volume (Greicius et al., 2008, Rykhlevskaia et al., 2008, Honey et al., 2009), this has not been well-investigated with regards to the molecular basis for these individual differences and its relationship to specific regional function or behaviour. The amygdala has been shown in both my dataset and previous studies to be larger in men than women. It has been noted by Goldstein that several studies show highest aromatase activity within the hypothalamus and amygdala, which also expresses the highest concentration of sex steroid receptors (Goldstein et al., 2001a). It is suggested that these asymmetries are modulated by sex androgen receptors(Hamann, 2005, Baas et al., 2004). Therefore I looked selectively at grey matter (GM) volumes of brain areas that co-vary with amygdala volume or left and right amygdala volume ratio. I then examined these derived regions in males and females for correlations with androgen receptor (AR) CAG, oestrogen receptor alpha (ESR1) TA, oestrogen receptor beta (ESR2) CA and aromatase (CYP19) TTTA repeat lengths.

4.2.2 Methods

T1 weighted 3D-MDEFT MRI images were taken from previously scanned healthy volunteers at the Wellcome Trust Centre for Neuroimaging. (248 Caucasians of European ancestry-125 male, 123 female). They were genotyped as described in the methods chapter. The images were segmented and normalised with DARTEL on SPM5 then smoothed for VBM. Amygdala volumes were generated as a ROI, and used as a covariate of interest to identify regions that differ significantly between males and females in the degree to which they vary with amygdala volume, with a statistical threshold of $p<0.001$ uncorrected and a cluster extent of more than 100 voxels. Individual GM densities obtained from the identified regions were divided by total amygdala volume and tested for an association with genotype across sex, within each sex and for a sex interaction. This ‘amygdala ratio’ provides a valid measure of the trophic relationship between the two regions that is equivalent to the beta derived in structural covariance.

4.2.3 Results

4.2.3.1 Regions showing significantly greater amygdala volume covariance in females.

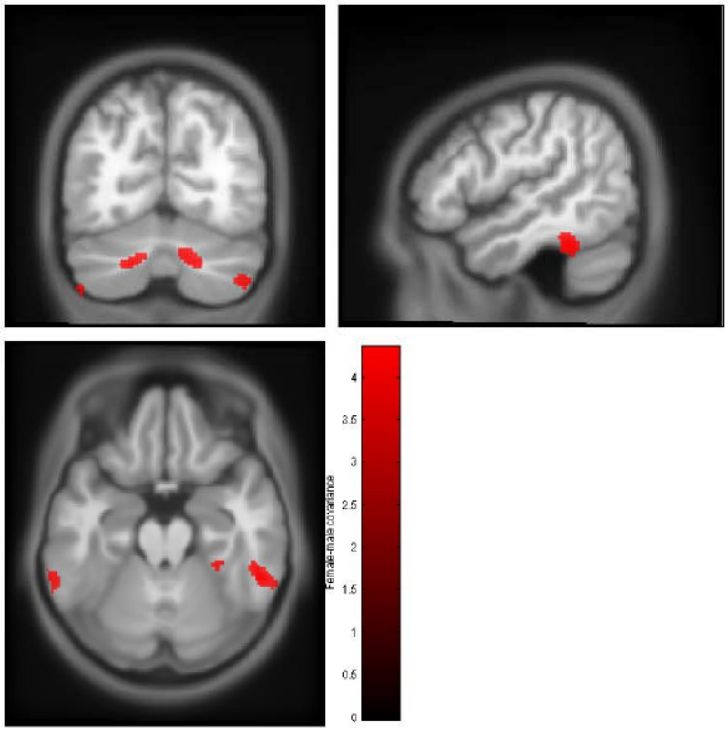


Figure 4.1 Regions showing significantly greater amygdala volume covariance in females than males. T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

The bilateral deep cerebellar nuclei and bilateral posterior-inferior temporal gyri covaried positively in females and negatively in males with amygdala volume.

4.2.3.2 Regions showing significantly greater amygdala volume covariance in males.

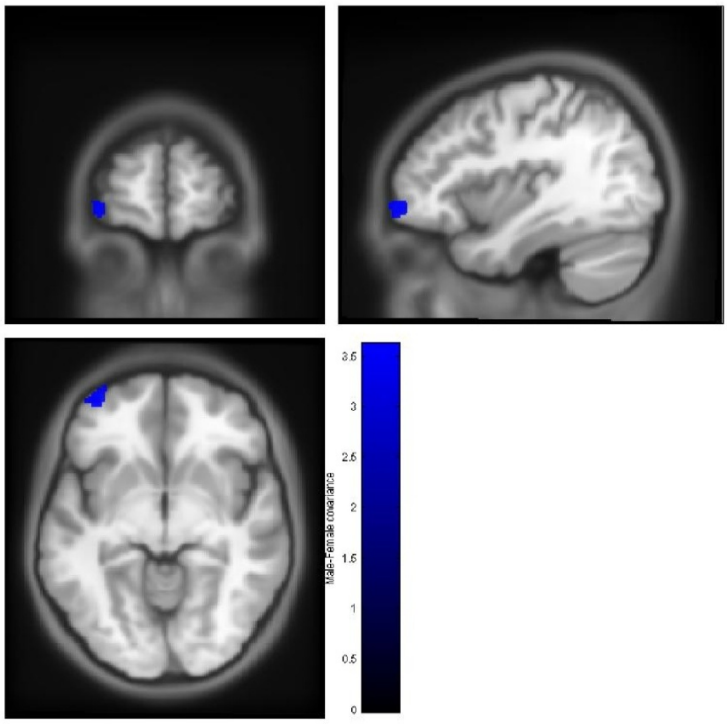


Figure 4.2 Regions showing significantly greater amygdala volume covariance in males than females. T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

Left lateral orbital gyrus volume covaried positively in males and negatively in females with amygdala volume.

4.2.3.3 Regions showing significant sex interaction with asymmetry of amygdala covariance.

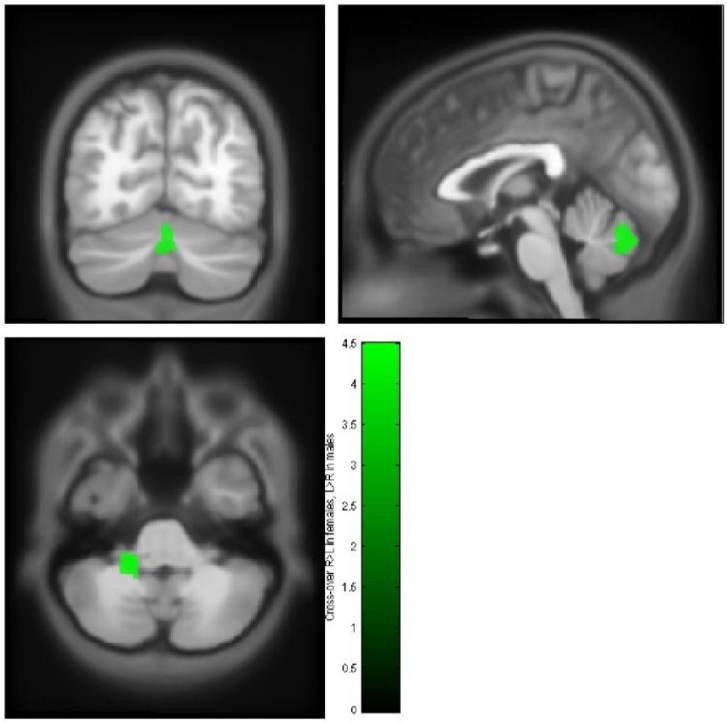


Figure 4.3 Regions with a significant difference in covariance with right over left amygdala volume ratio between males and females. T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

The left-right amygdala ratio identified significant female positive, male negative covariance in the cerebellar vermis, left inferior cerebellar peduncle.

4.2.3.4 Influences of sex hormone-related genetic polymorphisms on sexually dimorphic amygdala covariance.

Structure	Right posterior inferior temporal gyrus	Left posterior inferior temporal gyrus	Left deep cerebellar nuclei
Sex	Male	Female	Female
Direction of Amygdala Covariance	Negative with volume	Positive with volume	Positive with volume
Genotype	AR	AR	ESR1
Correlation	-0.292	0.154	-0.165
Significance (one tailed)	0.0005*	0.045	0.034

Table 4.1 Significant genetic influences on amygdala covariance. Bivariate correlations were performed between ESR1(TA)n, AR(CAG)n, ESR2(CA)n and CYP19(TTTA)n genotypes and extracted measures as a fraction of amygdala volume. Associations surviving correction for multiple comparison are marked with an *.

These areas were tested for significant correlations with genotype. AR(CAG)n was negatively correlated with the right posterior inferior temporal gyrus over amygdala GM volume ratio and positively with left posterior inferior temporal gyrus over amygdala GM volume ratio. ESR1(TA)n was negatively correlated with left deep cerebellar nuclei over amygdala GM volume ratio.

4.2.4 Conclusion

Structural regions which co-vary significantly with amygdala volume and volumetric ratio between sexes were identified. Also, I have demonstrated significant correlations between androgen-receptor CAG length with a positive covariance of the left posterior inferior temporal gyrus in females, and the opposite effect in the contralateral homotrophic region in males. Additionally in females, the left deep cerebellar nuclei region is positively associated with ESR1 length. The emotive facial processing networks are common to all these findings (Schutter and Van Honk, 2005, Hofer et al., 2006).

In women, responses to emotive visual stimuli and emotive recall are stronger in the left amygdala (Kilpatrick et al., 2006, Gasbarri et al., 2006). Additionally the enhanced recall of emotive material is lost in female left temporal lobectomy patients (Tomaz et al., 2003). I have demonstrated significant left hemispheric positive structural covariants with amygdala volume in these networks that are positively correlated with AR length in women. These findings suggest, as with previous studies, that the observed asymmetries in emotive processing may be driven by sex hormone receptors.

4.3 Sex differences in white matter volume and fractional anisotropy

Having discovered a genetic basis for sexual dimorphism in structural covariance, I then investigated the white matter itself both in terms of volumetric differences as well as integrity as indicated by fractional anisotropy using a voxel-wise approach.

Sexual dimorphism of white matter volume has been characterised in a number of studies, albeit less extensively than in grey matter. Lenroot et al. found that white matter volume, particularly in the frontal lobe, follows a steeper rate of increase in males during puberty

than girls (Lenroot et al., 2007). This was replicated by Perrin et al who showed age-related increases in white matter volume and decreases in magnetisation transfer ratio (MTR) in male adolescents relatively absent in female adolescents, who only appear to show increases in MTR in their frontal lobes (Perrin et al., 2009). Regional white matter differences in sexes are less well described than are grey matter differences perhaps due to the difficulty in making valid comparisons about homologous structures. On T1-weighted scans, white matter appears relatively homogenous and on the interior lacks clear features such as sulci and gyri found in cortex. Good et al have described larger white matter volume in the anterior temporal lobes and internal capsules in males, and larger white matter volume in the posterior frontal lobes and optic radiation in females (Good et al., 2001).. Reports on corpus callosal differences have been relatively mixed. For example, Lacoste-Utamsing and Holloway describe larger corpus callosa in males than females. Allen et al. describe shape-based sex differences in the corpus callosum between males and females despite no differences in overall size, with the splenium being more bulbous in females and more tubular in males (Allen and Gorski, 1992).

Diffusion tensor imaging has recently become more popular for investigating white matter properties and structural connectivity. Measures such as fractional anisotropy (FA) are thought to reflect the integrity, axonal properties and degree of myelination of white matter tracts that may help to understand the basis for volumetric differences in white matter. Additionally, while a handful of DTI studies report sex differences, the numbers of participants included in these comparisons have been relatively small. Shin et al found decreased FA in the corpus callosum of 15 females compared to 15 males (2005). This finding was replicated by a larger sample of 67 individuals by Westerhausen et al. On the other hand, studies such as (Eluvathingal 2007) in about 30 normal children and adolescents found no differences in FA or axial diffusivity between sexes, but lower transverse diffusivity in the inferior longitudinal fasciculus and the right inferior fronto-occipital fasciculus in girls compared to boys interpreted as indicative of higher myelin content in girls by the authors.

White matter volume increases rapidly during puberty in boys, and less so in girls. A recent study in 408 developing adolescents showed that males with a lower number of CAG repeats in the AR gene or higher expression of the androgen receptor had larger testosterone-related increases in white matter volume with age. As there was no association with magnetisation transfer ratio, the authors suggest that the changes are unrelated to myelination but may be instead due to increases in axonal diameter (Perrin, 2008).

In this section, I first investigate sexual dimorphism in white matter volume, structural connectivity and fractional anisotropy. I go on to compare regions of sexual dimorphism in white matter with regions influenced by each of the sex hormone-related gene polymorphisms in turn, finally considering sex by genotype interactions.

4.3.1 Methods

4.3.1.1 Image acquisition

Image preprocessing was performed for GM, WM and FA VBM as described in the methods section

Genotyping was performed as described in the previous section.

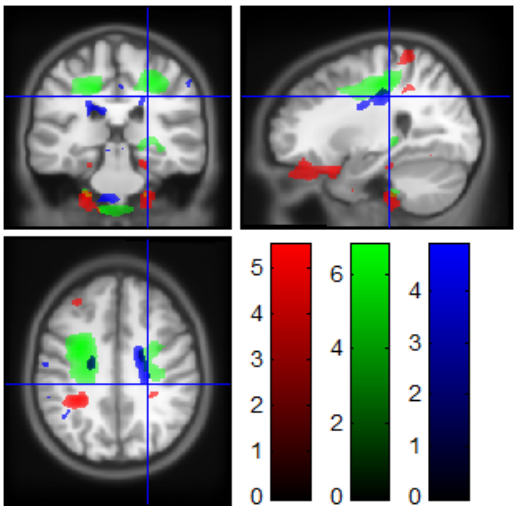
Analysis was performed by voxel-wise mapping of individual differences in grey and white matter density as well as fractional anisotropy across the brain. Comparisons were first made between sexes followed by each polymorphism and finally looking for a sex interaction in the association with each polymorphism. T-statistic images were overlaid on the warped averaged template in MNI space and MNI-DTI template with red representing

comparisons of grey matter, green representing white matter and blue representing fractional anisotropy.

4.3.2 Results

Having established regions of grey matter within the brain, I investigated the extent to which their concomitant white matter circuits were similarly influenced by sex.

4.3.2.1 Regions significantly greater in females in volume and FA.



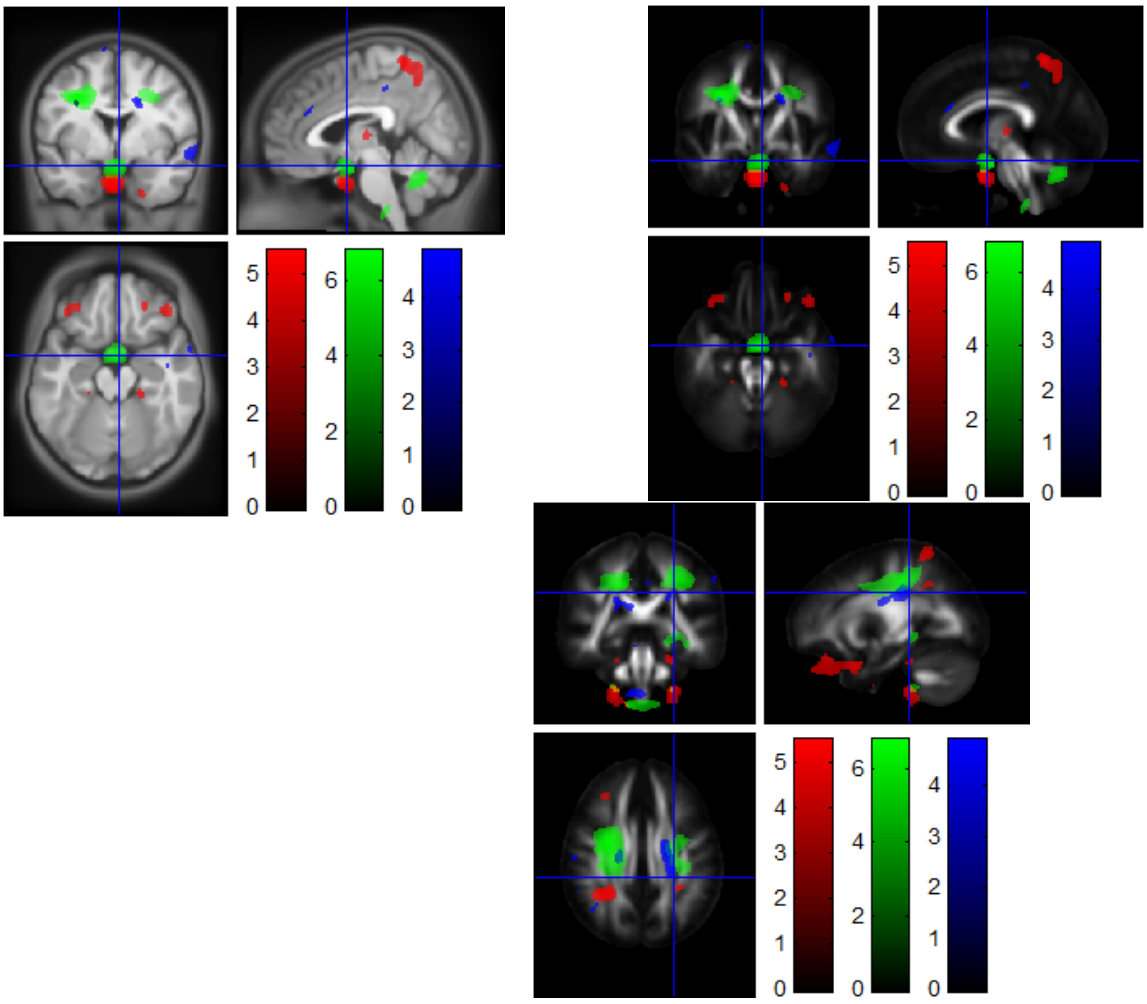


Figure 4.4 Regions greater in females than males in GM volume (Red), WM volume (Red) and FA (Blue). T-statistic maps thresholded at p>0.05 fwe-corrected were overlaid on the average of individual T1-weighted and DTI images warped together into MNI space.

Females have larger volume in the pituitary, brain stem, somatosensory cortex and the superior longitudinal fasciculus. Within the pituitary, grey matter volume is larger as was the anterior commissure directly superior without accompanying differences in fractional anisotropy. Within the brain stem on the other hand, white matter tracts inferior to the pons were both larger and demonstrated increased FA in women, with accompanying increased grey matter directly lateral. The superior longitudinal fasciculus was also increased in women with increased FA and increased grey matter in the contiguous grey matter in the somatosensory and superior parietal cortex.

4.3.2.2 Regions significantly greater in males in volume and FA.

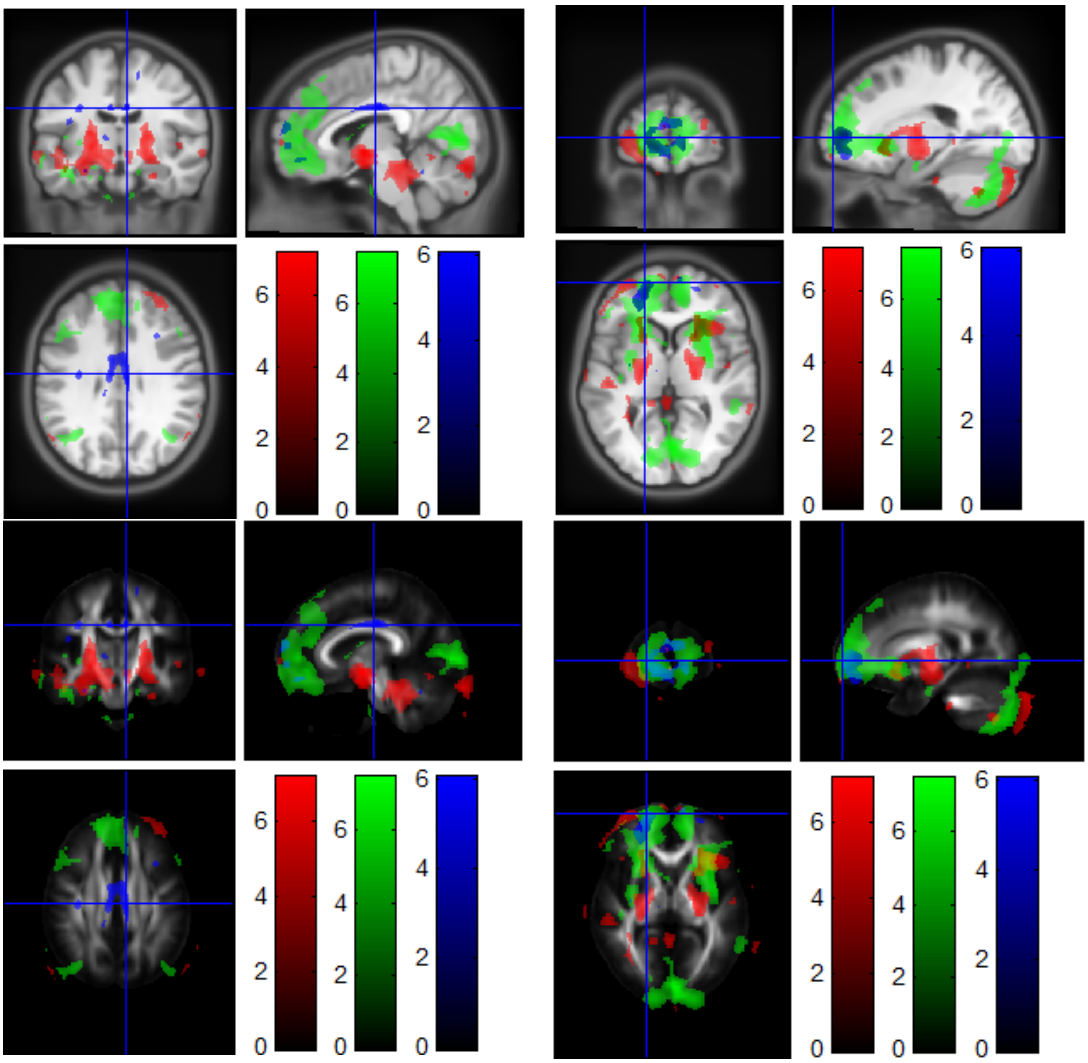


Figure 4.5 Regions greater in females than males in GM volume (Red), WM volume (Red) and FA (Blue). T-statistic maps thresholded at p>0.05 fwe-corrected were overlaid on the average of individual T1-weighted and DTI images warped together into MNI space.

In males, as compared to females, larger white matter volume accompanied adjacent regions of larger grey matter volume in the temporal lobes, insula, midbrain and cerebellum. Increased volume of the striatum was also accompanied by increased volume of the internal and external capsules in males as compared to females. However increased hypothalamic volume in males did not appear to be accompanied by increased volume of the white matter tracts leading to it, while there appeared to be isolated increases in medial

frontal and occipital white matter comprising the branches from the inferior fronto-occipital fasciculus. Males had greater fractional anisotropy in the cingulum without associated volume increases as well as the rostral frontal lobe associated with the white matter volume increases.

4.3.2.3 Regions significantly associated with AR(CAG)n

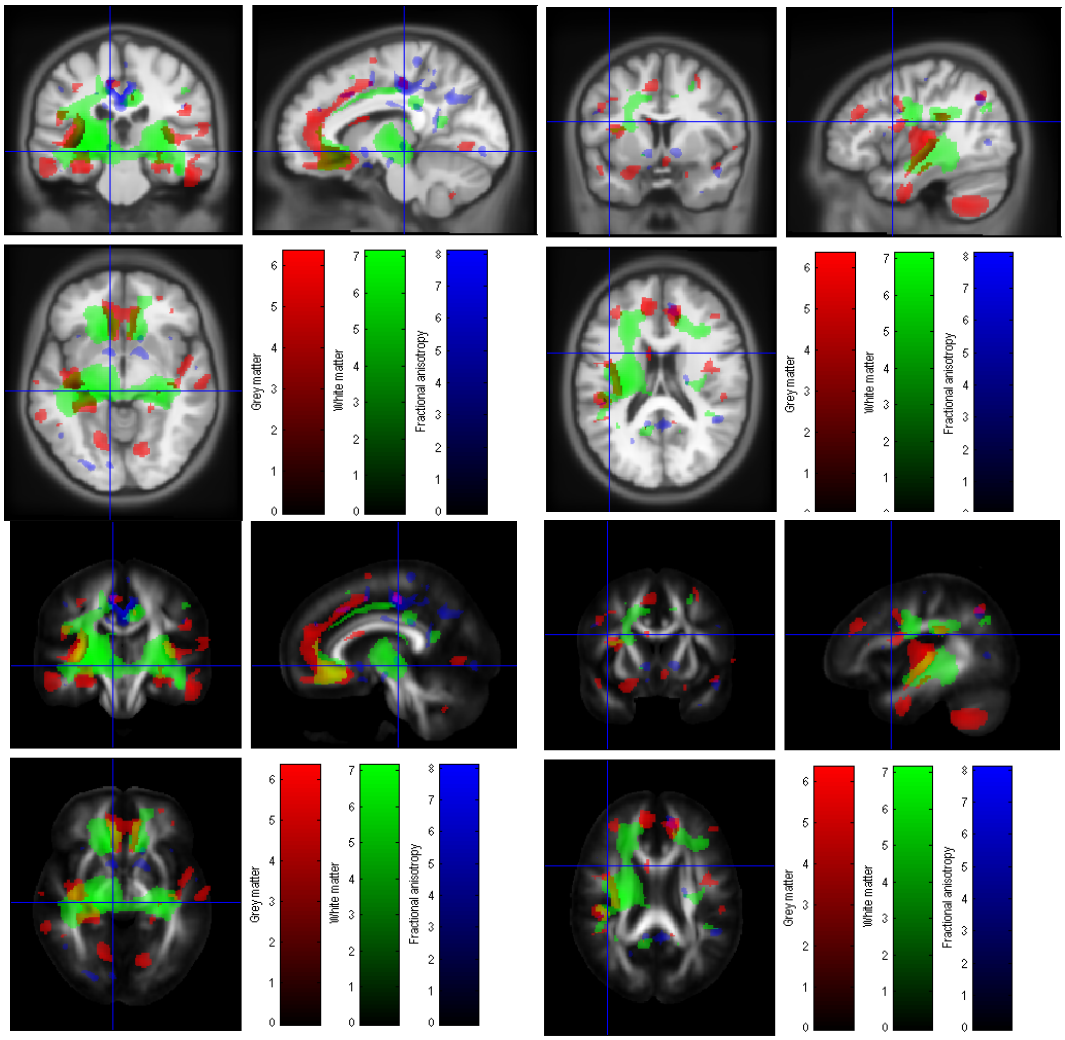


Figure 4.6 Regions positively associated with AR(CAG)n in GM volume (Red), WM volume (Red) and FA (Blue). T-statistic maps thresholded at p>0.05 fwe-corrected were overlaid on the average of individual T1-weighted and DTI images warped together into MNI space.

Having established the grey matter regions associated with AR CAG repeat length, I sought to determine whether concomitant white matter tracts could be similarly influenced in either volume or fractional anisotropy. Consistently individuals with higher CAG repeats showed larger GM volume, larger WM volume and increased FA. Within the white matter, higher CAG repeats were associated with increased volume in the subgenual ACC,

thalamus, superior longitudinal fasciculus, inferior fronto-occipital fasciculus, uncinate, cingulum and fornix. Increased FA was observed at the hypothalamus near the anterior commissure and posterior corpus callosum.

4.3.2.4 Regions significantly associated with ESR1(TA)n

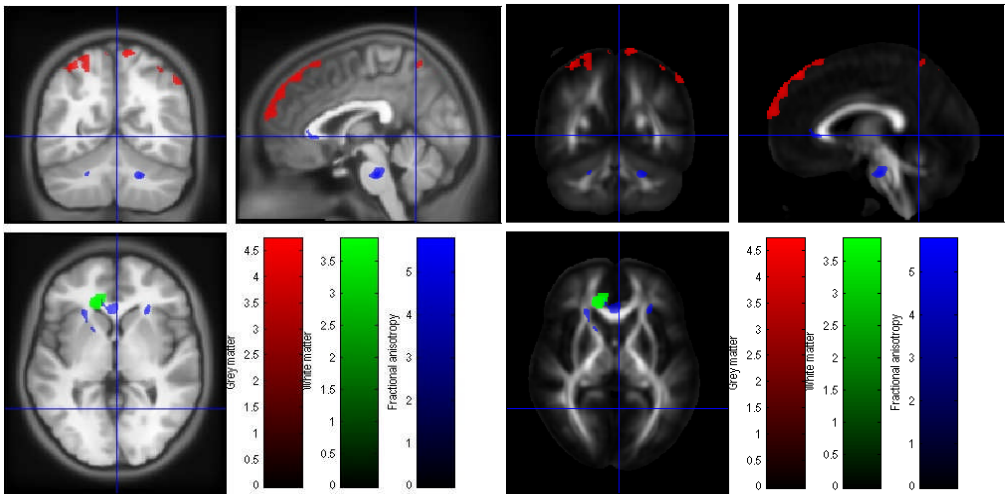


Figure 4.7 Regions where ESR1(TA)n high repeat homozygotes > heterozygotes and low repeat homozygotes in GM volume (Red), WM volume (Red) and FA (Blue). T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted and DTI images warped together into MNI space.

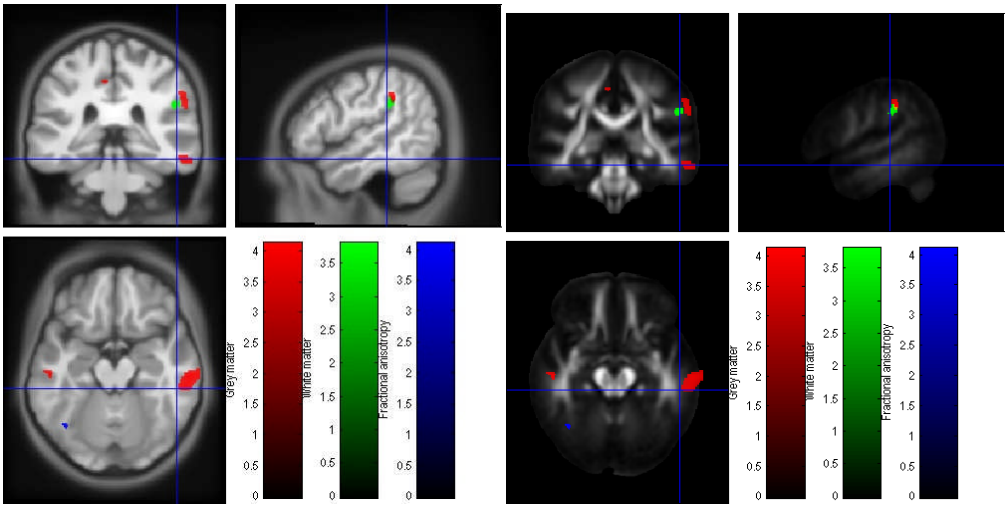


Figure 4.8 Regions where ESR1(TA)n low repeat homozygotes and heterozygotes > high repeats in GM volume (Red), WM volume (Red) and FA (Blue). T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted and DTI images warped together into MNI space.

White matter associations with ESR1 TA repeats were less apparent than with AR CAG repeats. High repeat homozygotes showed greater fractional anisotropy in the middle cerebellar peduncle and corticopontine tract as well as the anterior corpus callosum as compared the low repeat homozygotes and heterozygotes. The left genu of the anterior corpus callosum also showed greater white matter volume in high repeat homozygotes. These areas were separate and are not known to be related to the grey matter regions implicated in the fronto-parietal cortex.

On the other hand low repeat homozygotes and heterozygotes demonstrated greater white matter volume in the superior longitudinal fasciculus at the temporoparietal junction adjacent to a region associated with increased grey matter volume, but without any associated FA increases.

4.3.2.5 Regions significantly associated with ESR2(CA)n

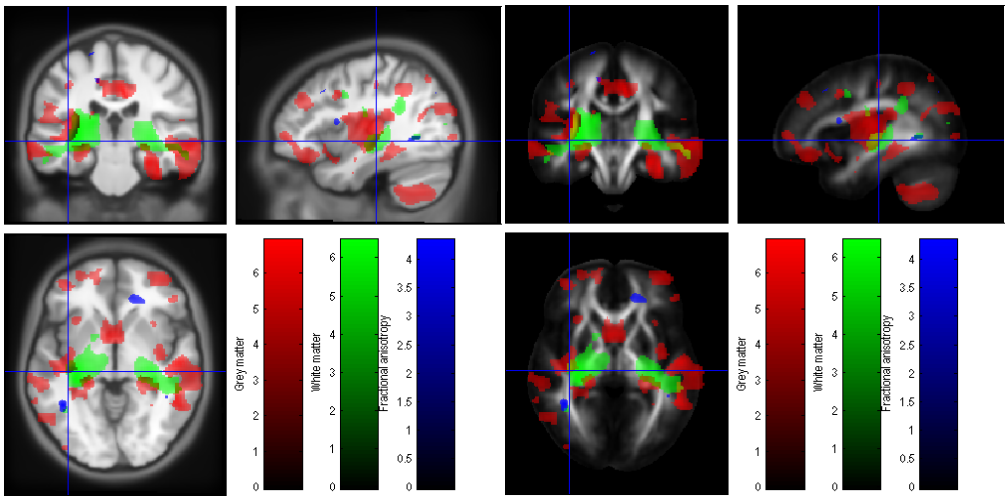


Figure 4.9 Regions in females where ESR2(CA)n low repeat homozygotes and heterozygotes > high repeats in GM volume (Red), WM volume (Green) and FA (Blue). T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted and DTI images warped together into MNI space.

With ESR2 CA repeat length, there were only white matter associations in females and not in males. White matter volume increases were visible in the uncinate and inferior longitudinal fasciculus and posterior limb of the internal capsule with no associated FA differences in female low repeat homozygotes and carriers as compared to female high repeat homozygotes. This was closely associated with the temporal cortex and insular grey matter differences.

4.3.2.6 Regions significantly associated with CYP19(TTTA)n

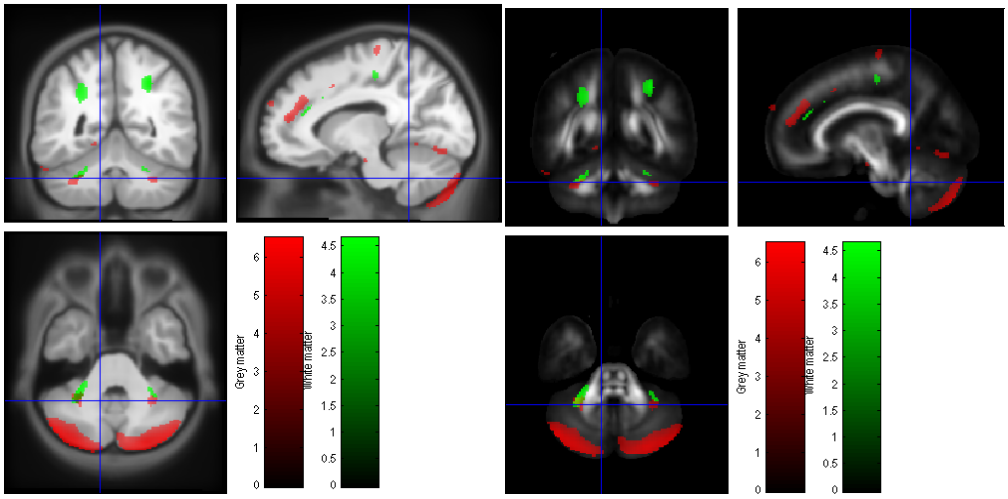


Figure 4.10 Regions with a significant sex by genotype interaction with CYP19(TTTA)n in GM volume (Red) and WM volume (Green). Associations were sought where the male correlation with increasing repeats were significantly larger than female correlation with increasing repeats. T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted and DTI images warped together into MNI space.

While there were no direct associations in grey or white matter volume with CYP19 TTTA repeats, the volume of a number of regions showed a significant sex by genotype interaction with male high repeat homozygotes over male heterozygotes or low repeat homozygotes showing significantly greater volume increases than female high repeat homozygotes over female heterozygotes or low repeat homozygotes. This was observed in both the grey and white matter of the cingulum, middle cerebellar peduncle with the posterior cerebellar hemisphere and the somatosensory cortex with the adjacent superior longitudinal fasciculus.

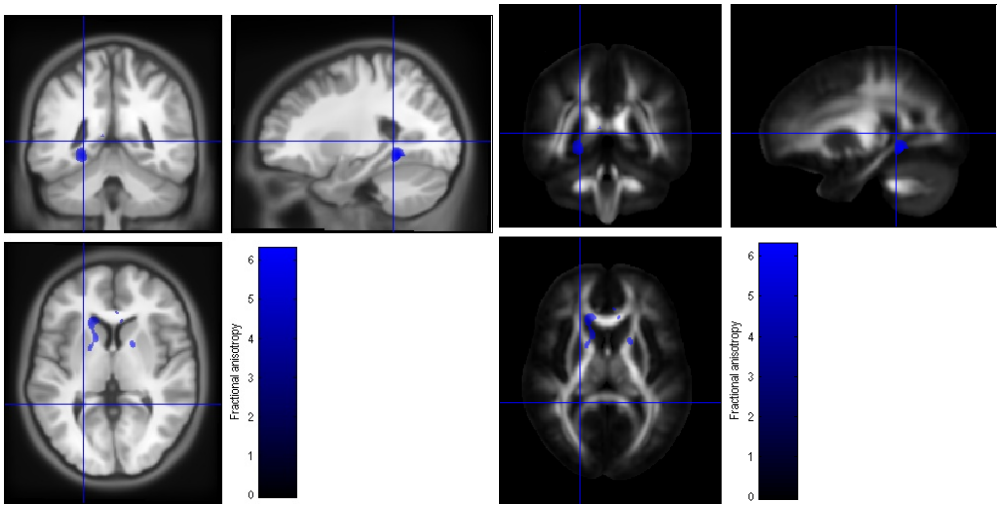


Figure 4.11 Regions with significantly greater FA (blue) in CYP19(TTTA)n high repeat homozygotes as compared to heterozygotes or low repeat homozygotes. T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted and DTI images warped together into MNI space.

On the other hand, differences in FA were apparent in the direct comparison, with high repeat homozygotes for CYP19 TTTA having greater FA in the left inferior-posterior cingulum adjacent to the fusiform gyrus and the anterior limb of the internal capsule.

No significant associations were observed between 2D:4D and any white matter regions.

4.3.2.3 Regional summary

Brain region	GM vol	WM vol	FA	AR	ESR1	ESR2	CYP19
Temporal lobe	M>F	M>F	Nil	GM, WM, +	GM, -	GM, WM, females -	Nil
Subcortical areas	Putamen, Thalamus, M>F	Intern. and external capsules M>F	Nil	WM intern capsule, thal, +	FA: ant int caps. +	GM thal, puta. WM Post Int. caps. F -	FA: ant. internal capsule
Somatosensory	SS cortex F>M	SLF, F>M	SLF, F>M	WM, FA +	Nil	Nil	GM, WM, sex int.. M+
Medial frontal lobe	BA10 M>F	Medial frontal, rIFOF, M>F	Rostral IFOF, cing. M>F	GM,WM, medial PFC, cing. +	GM, dm-PFC, +	BA10 GM in females -	Ant cing. GM, WM Sex int.. M+
Middle cingulum	Nil	Nil	M>F	WM,FA, +	Nil	Nil	Nil
Cerebellum	Post. Cerebellum M>F	Post. cerebell, M>F, Deep nuclei F>M	Nil	GM post cerebell +	Nil	GM, vent. Cerebellum. F -	GM post. Hemisph. Sex interact. M+
Hypothal.	M>F	Nil	Nil	GM, FA, +	Nil	GM, F –	Nil
Pituitary	F>M	AC, infundib., F>M	AC, F>M	Nil	Nil	Nil	Nil
Occipital lobe	Nil	Occipital cIFOF, M>F	Nil	Nil	Nil	Nil	Nil

Table 4.2 Summary by region of gene associations with each tissue type. First three columns indicate the direction of sex differences. + or – signs indicate positive and negative gene associations with repeats respectively. F indicates that associations were only in females with ESR2(CA)n, while M+ indicates the direction of the sex association with CYP19(TTTA)n.

As mentioned in the chapter on grey matter, the temporal lobes were larger in males than females. This was similarly evident in the insula in grey matter volume and uncinate fasciculus and fornix in white matter volume. There were no associated sex differences in FA in the temporal lobe. Much of the temporal cortex and insula in grey matter and uncinate, fornix and temporal stem in white matter volume were positively associated with AR CAG repeats. No white matter differences accompanied the grey matter association with ESR1 in the temporal cortex. ESR2 CA repeats were similarly negatively associated with temporal and insular grey matter and uncinate and temporal stem white matter in women. No association was observed with CYP19 TTTA repeats.

The GM volume of the putamen and thalamus and WM volume of the internal and external capsules were larger in males than females. WM volume in the internal capsule and thalamus was positively associated with AR CAG repeats. FA in the anterior limb of the internal capsule was positively associated with ESR1 TA repeats. The grey matter volume in the thalamus and putamen and the white matter in the posterior internal capsule were negatively associated with ESR2 CA repeats in females. The anterior limb of the internal capsule was also positively associated with CYP19 TTTA repeats.

The somatosensory cortex and adjacent branches of the superior longitudinal fasciculus were larger in females than males and had increased FA in females. It was positively associated with repeat length of the AR CAG polymorphism regardless of sex in white matter volume and FA. It also demonstrated a sex interaction with CYP19 TTTA genotype, where longer repeats associated with increased GM and WM volume in males and decreased GM and WM volume in females.

The medial and rostral aspect of the frontal lobe was larger in males than females. This was apparent in medial prefrontal cortex, as well as the adjacent white matter and was accompanied by increased FA. Only the ventral aspect in the orbitofrontal cortex was larger in females. In the frontomedial region, FA and white matter volume was positively associated the AR CAG repeats, while grey matter volume was negatively associated with

ESR2 CA repeats in females. On the other hand GM and WM volume demonstrated a sex interaction with greater positive association with CYP19 TTTA repeats in males around the anterior cingulate. The middle cingulum also larger in males, was positively associated in white matter and FA with AR CAG repeats.

The cerebellum was larger in white and grey matter in males at the posterior aspect of its hemispheres, but was larger at the deep cerebellar nuclei in women. The same grey matter region was positively associated with AR CAG repeats, negatively with ESR2 CA repeats in females and showed a sex interaction with CYP19 TTTA repeats with more positive correlation in men.

No white matter differences appeared to accompany the hypothalamic differences and associations in grey matter volume and no genetic associations were found to accompany the sex differences in the occipital, pituitary and anterior commissure.

4.3.3 Discussion

Overall regions with significant association with sex hormone-related polymorphisms were also sexually dimorphic and the majority of sexually dimorphic regions had gene associations. GM and WM volume as well as FA were generally positively associated with AR CAG repeats, indicating decreased expression of androgen receptor, negatively associated with ESR2 CA repeats in females, circumstantially indicating increased expression of oestrogen receptor beta, and showed a sex interaction with CYP19 TTTA repeats with males showing a positive and females a negative association. ESR1 repeats caused increases in some regions and decreases in others. The findings with AR CAG and ESR2 CA repeats are in agreement with those in the previous chapter suggesting that increased expression of the oestrogen receptor beta in females, who have a mileux of high bioavailable oestrogen and higher overall levels of oestrogen receptor expression, and decreased expression of unbound androgen receptor have a trophic influence.

As discussed in the introductory chapter, aromatase genotype appears to have opposing influences on bone mineral density between men and women as it does on regional brain volume. This might be explained by differential availabilities of the sex hormones in those brain regions between men and women where a certain ratio might be optimal for development or the prevention of age-related atrophy. Also if relative sex hormone receptor expression differs between sexes and the overall pool of androgens and oestrogens is limited, then a certain proportion driven by aromatase expression could maximise receptor occupancy.

In this thesis, I only made regional comparisons on the basis of relative volume, after correcting for appropriate compartmental volumes such as overall GM volume for GM comparisons or white matter volume for WM comparisons. Thus where some studies might report differences that would vary with overall compartmental volume, they would not be significant in my analysis. While sex difference in grey matter volume has been fairly extensively studied, traditionally white matter analysis of sexual dimorphism has been confined to the corpus callosum, which in turn has been relatively equivocal with males having larger overall but smaller relative callosal volume. Shape differences between regions might additionally cause differences in sensitivity for a given region between methods.

In the temporal lobes, the increased volume of the grey matter and white matter in males as compared to females closely replicates previous VBM comparisons of sex difference in the brain (Good et al., 2001). The absence of FA changes suggests that this difference is likely morphological. The greatest differences were over the ventral aspect of the temporal lobe whereas the superior temporal gyrus especially posteriorly was actually larger in females. This relative distribution has been similarly observed in cortical thickness measurements (Sowell et al., 2007). ESR1 TA repeats were negatively correlated with GM and WM volume at the right temporo-parietal junction, the same region found to be larger in women, and with GM volume in the inferior temporal gyrus bilaterally, which was larger in men.

Low repeats have been associated with decreased bone mineral density and would be expected to relate to decreased expression of oestrogen receptor alpha, so it would appear that increased expression has an inhibitory effect on size of these regions that is ameliorated by relatively higher oestrogen levels in women at the temporo-parietal junction. On the other hand, it could be that the opposing effects of AR and ESR2 within the rest of the temporal lobe have a stronger influence resulting in the higher volume of those regions in men.

The somatosensory cortex is consistently larger in females in volume (Goldstein et al., 2001a),, VBM measures of grey matter density (Good et al., 2001). and cortical thickness (Sowell et al., 2007). Additionally a number of studies have found that females have greater sensitivity to pain and finer haptic perception in tasks of tactile discrimination and sensitivity (Ellermeier and Westphal, 1995, Feine et al., 1991, Maccoby and Jacklin, 1974). It has been recently reported that finger size accounts for these sex differences in tactile discrimination (Peters et al., 2009), suggesting that experience-dependent plasticity or modulation shapes these differences with changes in the long range connections along the white matter tract leading to the cortical area. However, my finding of an association with AR CAG and CYP19 TTTA would imply that there is also a hormonal influence on this dimorphism potentially acting directly on the development of this region. Superior longitudinal fasciculus I, comprising the dorsal component of the superior longitudinal fasciculus was the specific component of the white matter tract involved and originates in the superior and medial parietal passes by the cingulate sulcus and terminates in the dorsal and medial frontal cortex (Wakana et al., 2004, Makris et al., 2005).

As found in other studies, the frontomedial and rostral prefrontal cortex was larger in males, while the ventral prefrontal cortex such as the lateral orbital gyrus was larger in females (Cosgrove et al., 2007). The ventral regions did not differ in white matter volume or FA between sexes nor were they associated with any sex hormone-related polymorphism. On the other hand the frontomedial region also had larger WM volume and increased FA in males, and was associated with AR CAG in FA and WM volume and with ESR2 CA repeats in GM volume in females. The disparate genetic association between

WM and GM is interesting and imply that the mechanisms of action differ between tissue types with ESR2 possibly acting on cells other than the large pyramidal neurons that provide long range projections. The frontomedial cortex is thought to be involved in mentalising and theory of mind. Some studies report early overgrowth of the frontal lobe in young children with autism with later slowing (Carper and Courchesne, 2000, Carper et al., 2002, Carper and Courchesne, 2005) and autism has been described as an extreme male phenotype with a link to uterine testosterone levels(Baron-Cohen et al., 2005).

Within the cerebellum, the anterior edge of the cerebellar nuclei or tonsil appeared to be greater in GM volume, WM volume and FA in females than males, but had no association with the investigated genetic polymorphisms. As this area abuts the fourth ventricle, there is the potential for artefact, although it would be unclear why this artefact would be associated with sex. The posterior lobe mostly inferiorly was larger in both GM and WM volume without any FA association. GM volume of the inferior posterior lobe was similarly associated with polymorphisms of AR CAG, ESR2 CA in females and CYP19 as a sex interaction as has been found in other regions larger in males, but these polymorphisms did not appear to influence WM volume in the cerebellum.

Thus it would appear the AR CAG, ESR2 CA and CYP19 TTTA influenced a set of sexually dimorphic regions including the temporal lobe, somatosensory areas, medial prefrontal lobe, thalamus, internal capsule and cerebellum. Many of these regions have known connections to one another such as between the superior parietal including the somatosensory and the medial prefrontal, as well as between the cerebellum and prefrontal via the pons and thalamus.

4.4 Normal variation in fronto-occipital circuitry and cerebellar structure with an autism-associated polymorphism of CNTNAP2

4.4.1 Introduction

Having described the contributions of sex hormone receptor genes to sexual dimorphism in structural brain circuitry, I then sought to investigate whether there was a relationship between

Autism is a developmental disorder defined by impaired social interaction and communication, and repetitive behaviours (Lord et al., 1994). However autism has been found to be heterogeneous in both its phenotypic manifestation and genetic aetiology (Bill and Geschwind, 2009). Rare gene mutations and de novo copy number variants account for much of the variance in the disorder (Sebat et al., 2007). Thus the need to define the nature of genetic populations and genes accounting for different phenotypic expressions of these behaviours is pressing. Autism-associated candidate polymorphisms are well suited for this approach. Autism has a heritability of over 70% (Bailey et al., 1995), while volumetric measures of the brain are as much as 90% heritable (Toga and Thompson, 2005). Autism constitutes a spectrum encompassing mild behavioural impairment that is compensated before the onset of adulthood (Wing, 1988) and relevant traits such as the autistic-spectrum quotient (Baron-Cohen et al., 2001) and the ability to perform tests of theory of mind (Baron-Cohen et al., 1996) are continuous within the normal population. Indeed significant morphological differences can be observed in the cortex of parents of ASD patients (Peterson et al., 2006).

Contactin (CNTN)-associated protein-2 (CNTNAP2) has been associated with ASD, epilepsy and schizophrenia (Strauss et al., 2006, Friedman et al., 2007, Burbach and van der Zwaag, 2009). A number of studies have found that polymorphism in CNTNAP2 confers

risk for ASD (Alarcón et al., 2008, Arking et al., 2008, Bakkaloglu et al., 2008, Rossi et al., 2008). CNTNAP2 polymorphism influences FOXP2 expression accounting for the development of specific language impairment (Vernes et al., 2008). CNTNAP2 encodes contactin-associated protein-like 2 (Caspr2), a single-pass trans-membrane protein that is a member of the neurexin superfamily (Baumgartner et al., 1996), which colocalises with voltage-activated K1 channels (Kv1) at the nodes of Ranvier (Poliak et al., 1999) and is necessary for their function (Poliak et al., 2003). Finally CNTNAP2 is important in the migrating neuroblast during cortical development, which underpins laminar organisation (Strauss et al., 2006) and the CNTNAP2 transcript is confined to developing frontotemporal-subcortical circuits known to be critical for executive function within the fetus (Alarcón et al., 2008).

Due to the axonal location of CNTNAP2, I was particularly interested in studying white matter so used voxel-based comparisons of Fractional Anisotropy (FA) using Diffusion Tensor Imaging (DTI) (Basser et al., 1994) and Voxel-based morphometry (VBM) (Ashburner and Friston, 2000). While DTI was used to study the architecture of cerebral white matter, I chose to carry out VBM on both grey and white matter because autism has been associated with increased cortical grey to white matter ratio and decreased volume of those compartments beyond childhood (Courchesne et al., 2001, Acosta and Pearl, 2004). Several studies with VBM and DTI have already been performed on various autistic populations with a wide range of results. Both increases and decreases of grey and white matter volumes and FA have been observed. The variability of these results is unsurprising given that the studied populations differed both in age and in autistic diagnosis (Eigsti and Shapiro, 2003). However certain regions have been consistently implicated by VBM studies, for example the cerebellum (Abell et al., 1999, Boddaert et al., 2004, Neufang et al., 2008, Stanfield et al., 2008).

My approach was to investigate whether polymorphism in rs7794745 CNTNAP2 among normal people is associated with variation in brain structure (Arking et al., 2008). Other studies implicating CNTNAP in ASD have investigated its associations with specific facets of ASD (Alarcón et al., 2008, Vernes et al., 2008). I decided to investigate this particular

polymorphism because it is associated solely with a diagnosis of autism that fulfills strict criteria and because the result has been replicated across two family-based cohorts. The authors report a significantly increased risk for autism in male homozygotes for the risk allele but not in females or male carriers.

Therefore I hypothesized structural deficits in homozygotes for the CNTNAP2 rs7794745 risk allele compared to heterozygotes and non-risk carriers. Due to the higher occurrence of ASD in males (Bryson, 1996) and the male-specific association in ASD risk conferred by the polymorphism (Alarcón et al., 2008, Arking et al., 2008), I investigated whether there is also a sex by genotype interaction with the CNTNAP2 rs7794745 polymorphism.

4.4.2 Methods

Genotyping and Image preprocessing were performed as in the previous section.

4.4.2.1 Statistical analysis

In the analysis, I examined the main effect of genotype and interactions with sex by creating whole brain statistical parametric maps (SPMs) for regional grey and white matter volume, as well as FA. In the general linear model, sex, genotype and scanner acquisition were factors in the analysis with nuisance covariates for age and a global measure of either volume or FA respectively. Variables were orthogonalised using a Gram-Schmidt process implemented in SPM. I tested for both increases and decreases in grey and white matter volume and FA between TT, at risk homozygotes, and AT/AA, heterozygotes and major allele homozygotes. I performed sex interaction analyses after the GM, WM and FA analyses to examine whether within regions that were significantly different for male and female TT compared to AT and AA there was a significant difference between males and females. Although primary analysis compared TT with AT/AA as suggested by the initial association (Arking et al., 2008), I also tested in post-hoc secondary analysis for significant

differences between homozygotes for the major non-risk allele and carriers or carriers and homozygotes for the risk allele.

Cluster-level inference was used as it makes use of information from the local spatial neighbourhood, however in VBM assumptions of cluster stationarity often do not apply. Thus a cluster-level threshold of $p<0.05$ with non-stationary cluster extent correction (Hayasaka et al., 2004), after family-wise error correction for multiple comparisons across the brain was used. An initial cluster-forming threshold of $p<0.001$ was used to ensure validity of cluster level inference. I also report peak voxels within each cluster for spatial localisation and for future reference as there continues to be debate on best practice in cluster-level inference. The FA analysis included small volume corrections around regions demonstrating WM volume changes as I expected regions implicated through reductions in white matter volume to also exhibit changes in FA.

4.4.3 Results

There were several regions in the brain with significant differences in grey and white matter volumes and fractional anisotropy surviving correction for multiple comparisons across the brain. Furthermore, there was broad spatial correspondence between measures, with significant clusters localised within the cerebellum, occipital and frontal lobes. White matter deficits in tracts containing fronto-occipital connections and the thalamic projections to those frontal and occipital cortices implicate an overall cortical circuit. Although not tested explicitly, these bilateral reductions appeared more prominent on the right. I found that homozygotes for the risk allele had less white and grey matter as well as decreases in FA in these regions. In the cerebellum only grey matter was decreased in risk homozygotes. Detailed anatomical results are reported first by measure and then by brain region.

4.4.3.1 Analysis by modality

4.4.3.1.1 GM volume

There was decreased GM in risk homozygotes in the right frontal pole, fusiform gyri, V1 cortex, posterior cerebellar hemispheres and vermis.

4.4.3.1.2 WM volume

Risk homozygotes also had decreased WM volume in the posterior thalamic radiation bilaterally, the right caudal inferior fronto-occipital fasciculus and the right rostral cingulum.

The posterior thalamic radiation comprises the fibres from the posterior nuclei of the thalamus to the parietal and occipital cortices (Wakana et al., 2004). The inferior fronto-occipital fasciculus connects the frontal lobe and occipital lobe, projecting between lateral frontal cortex rostrally and areas such as the fusiform gyrus (Catani et al., 2002). The cingulum contains the projections from the anterior thalamic nucleus to the prefrontal cortex (Bürgel et al., 2006).

4.4.3.1.3 FA

Risk homozygotes showed no significant FA reductions, however in sex-specific analyses male risk homozygotes had decreased FA in the right rostral inferior fronto-occipital fasciculus and females in the right anterior thalamic radiation.

4.4.3.2 Regional results

4.4.3.2.1 Occipital lobe

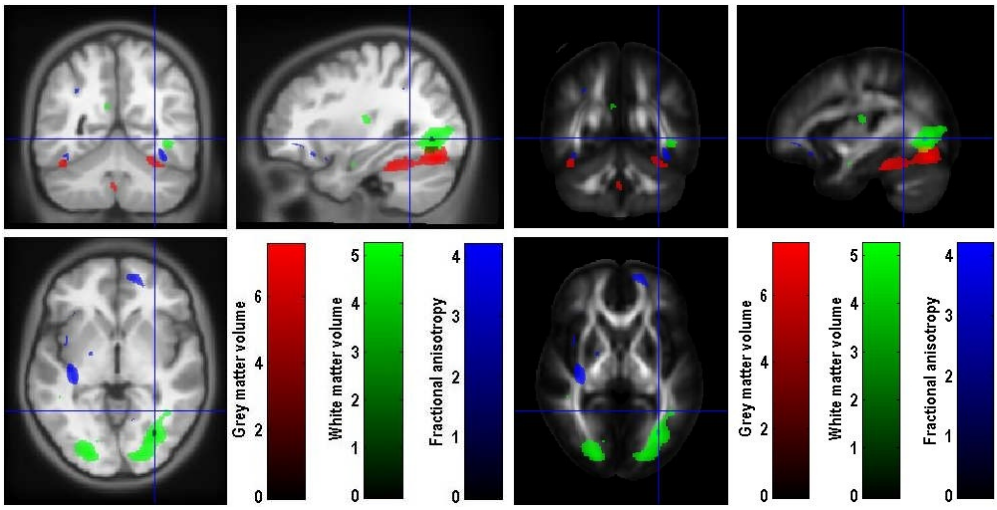


Figure 4.12 Occipital lobe reductions in CNTNAP2 risk homozygotes. GM volume (Red), WM volume (Green) and FA (Blue). T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted and DTI images warped together into MNI space.

Risk homozygotes had reduced GM volume in the fusiform gyrus bilaterally and reduced white matter volume in the right caudal inferior fronto-occipital fasciculus. Dorsally, risk homozygotes had reduced GM in the posterior occipital cortex and reduced WM volume and FA in the posterior thalamic radiation.

4.4.3.2.2 Cerebellum

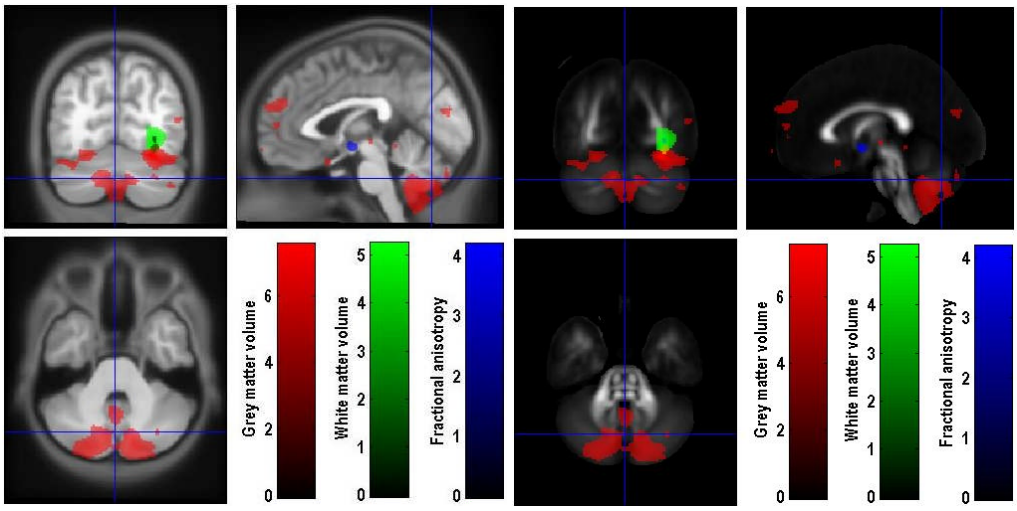


Figure 4.13 Cerebellum reductions in CNTNAP2 risk homozygotes. GM volume (Red), WM volume (Green) and FA (Blue). T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted and DTI images warped together into MNI space.

Within the cerebellum there were significantly reduced GM volumes in risk homozygotes in lobule VI of the left superior hemisphere and in crus I of the posterior hemisphere bilaterally. The vermis also had reduced grey matter volume. No reductions in WM were observed in the cerebellum.

4.4.3.2.3 Frontal lobe

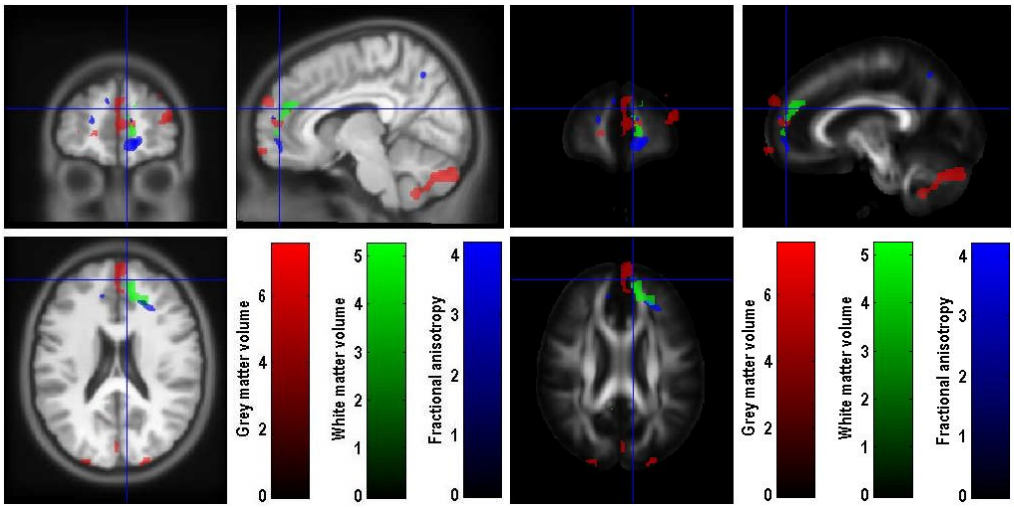


Figure 4.14 Frontal lobe reductions associated with CNTNAP2 risk allele in GM volume (Red), WM volume (Green) and FA (Blue). T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted and DTI images warped together into MNI space.

Significant reductions in GM volume in risk homozygotes were found in the right frontal pole and in WM volume in the right rostral cingulum. Similarly, reduction in FA can be seen bilaterally in which on the right appears closely associated with the white matter reduction.

4.4.3.3 Sex interaction

I then tested for a sex by genotype interaction, which was present in GM volume and FA but not in WM volume.

4.4.3.3.1 Grey matter

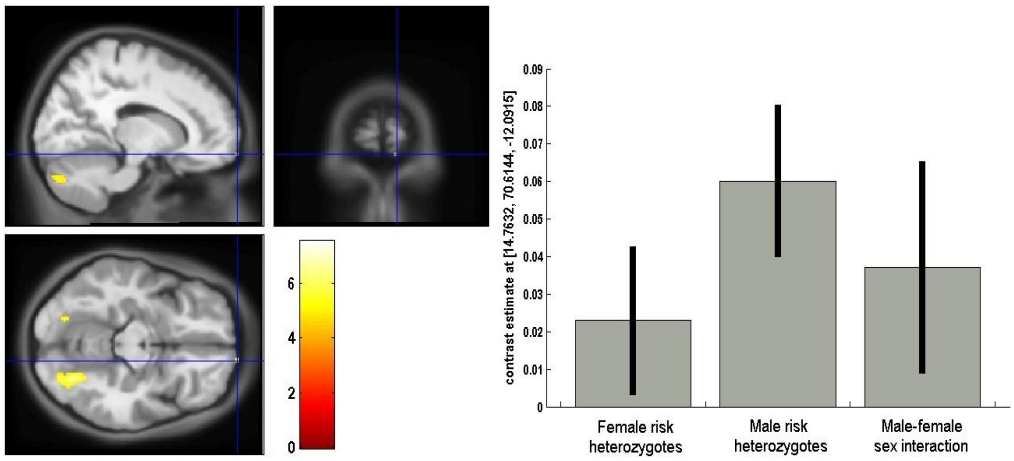


Figure 4.15 Regions showing significant sex by genotype interaction with the CNTNAP2 risk allele in GM volume. T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted warped together into MNI space.

Male risk homozygotes showed greater reduction of GM in the right frontal pole than did females.

4.4.3.3.2 FA

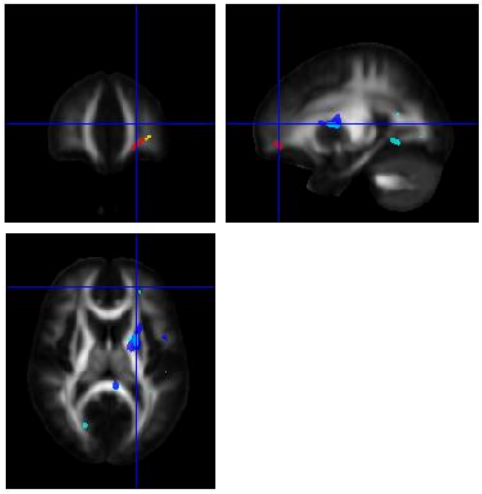


Figure 4.16 Regions showing sex-specific associations and a sex by genotype interaction with the CNTNAP2 risk allele in FA. Maps are displayed of FA reductions in risk allele homozygotes in males (blue), in females (red), and where reductions were significantly greater in males than females (cyan), and greater in females than males (yellow). T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted and DTI images warped together into MNI space.

Sex-specific analyses demonstrated significant cluster-level results in the right rostral inferior fronto-occipital fasciculus in male risk homozygotes and the right anterior thalamic radiation/putaminal head in female risk homozygotes compared with their respective non-risk homozygotes in FA. The same regions were significant when tested formally for a sex by genotype interaction.

4.4.4 Discussion

In this study, I discover significant regional reductions in GM volume, WM volume and FA as well as sex-specific reductions in fractional anisotropy of normal control subjects homozygous for the CNTNAP2 rs7794745 risk allele. Additional tests for significant differences between homozygotes for the major non-risk allele and carriers or carriers and homozygotes for the risk allele yielded negative results suggesting, as was found with disease risk, a recessive mechanism of action where the risk-allele is likely to confer a loss of function (Arking et al., 2008).

Surprisingly, these differences broadly occurred in both males and females despite the male-specific association of the polymorphism with disease risk and the known male bias in autistic prevalence. While cerebellar and occipital deficits were similar across sexes, GM volume in the right frontal pole and FA in the right rostral inferior fronto-occipital fasciculus showed greater reductions as compared to controls in male risk homozygotes than their female counterparts. Whereas in male risk homozygotes this was associated with inferior fronto-occipital fasciculus FA reductions, in female risk homozygotes the FA deficits appeared instead in the anterior thalamic radiation that is known to project to the right rostral prefrontal cortex. Could this frontal difference account for the disparity in phenotypic manifestation of the risk allele between sexes? While motor, language and perceptual differences in autism are significant, it has often been the prefrontal-associated cognitive functions such as mentalising, cognitive flexibility and social attention that are considered defining and it has been argued that long distance disconnection, but local over-connectivity of the frontal lobe could be a key deficit in autism (Courchesne and Pierce, 2005). It is known that females have more grey matter in the right inferior frontal gyrus and perhaps the developmental factor underlying this may also compensate for the effect of the risk allele (Good et al., 2001). Patients with Turner's syndrome, who are females lacking an X-chromosome and are also found to exhibit autistic features such as impaired recognition of fearful facial expressions, have unusually large orbitofrontal cortex, a region proximal to that implicated in the sex interaction in grey matter and projecting in part to the inferior fronto-occipital fasciculus in addition to the uncinate (Good et al., 2003). Thus an

interaction with an X-linked gene might mediate some of the sex differences in the effect of the risk allele. On the other hand, the anterior thalamic radiation/putaminal head showed greater reductions in FA in females risk homozygotes than their male counterparts. This region has been previously found to be reduced in white matter volume in male adolescents with ASD as compared to controls (Waiter et al., 2004). The anterior thalamic radiation is not broadly considered to be a region exhibiting sexual dimorphism so it would be interesting to determine how sex-related factors might influence the effect of CNTNAP2.

Of note, I observed no significant increases in grey matter volume, white matter volume or fractional anisotropy and instead concordant decreases in grey matter volume and white matter volume in regions previously implicated in autism. Given the localization of CNTNAP2's expressed protein, Caspr2, to the juxta-paranodal region of myelinated axons and an imputed role in scaffolding (Poliak et al., 1999, Poliak et al., 2003), one likely possibility is that retarded development or loss of axons might be driving concomitant impairments in grey matter. This might be the case if CNTNAP2 played a developmental role in the growth of axons or though axonal trafficking of neurotrophic factors. In support of this, transgenic knockout of CNTNAP2 or its binding partner was found not to cause cortical dysplasia, but prevented spatial clustering of potassium channels. This effect on ion channels could also impair axonal propagation of the action potential in some axons and lead to increased elimination during pruning in neuronal development, an epistatic interaction between developmental plasticity and the functional effects of the mutation. Such axon-driven mechanisms could explain the close concordance I find between adjacent grey and white matter reductions, although it is also suggested that Caspr2 is involved in neurogenesis and cortical histogenesis due to its role in cortical dysplasia (Strauss et al., 2006) and thus may have a direct effect on the development of grey matter. The reduction of FA was relatively less consistent, where often there was a trend towards reduction that did not survive multiple correction. A sample size for the FA analysis was 114 out of the 314 and a lack of power or sensitivity in the methodology can explain this. Additionally, it may be possible that myelination and axonal integrity remains relatively preserved in many of these areas despite neuronal loss or lack of neurogenesis, the latter a plausible outcome if the mutation had no degenerative effect.

I found regional reductions within the right prefrontal and bilaterally in the fusiform gyri, posterior occipital cortices and the cerebelli in risk homozygotes compared to heterozygotes and major allele homozygotes. In addition, to grey matter reductions within these regions, there were white matter reductions in the right inferior fronto-occipital fasciculus on both the frontal and occipital ends of the tract as well as the posterior thalamic radiation bilaterally and right rostral cingulum. Together with the hypothesis that impaired axonal development might drive these changes, these results suggest that this polymorphism of CNTNAP2 acts in part through disruption of the fronto-occipital connection. The thalamic projections to those regions also demonstrated deficits and it is interesting to note that both thalamocortical and intrahemispheric afferents project to neurons within layer IV providing a possible means by which deficits in IFOF could influence cortex and thalamocortical connections (Shepherd, 2004). CNTNAP2 could also feasibly influence this circuit, as in situ hybridisation in human foetal brains revealed that a CNTNAP2 transcript was confined to developing frontotemporal-subcortical circuits known to be vital for executive function (Alarcón et al., 2008).Age-related reductions in FA of the right IFOF have been shown to relate to the accuracy of face processing by perceptual discrimination, not as clearly apparent with non-face objects (Thomas et al., 2008). Additionally the fusiform face area, which is specifically activated in response to faces is located in the right fusiform gyrus and connected to the right IFOF (Kanwisher et al., 1997). It is underactive in ASD (Schultz et al., 2003) and facial perception is impaired in ASD (Grelotti et al., 2002). Despite the clear connection to autism through face-processing, anatomical abnormalities in the fusiform gyrus and occipital lobe are less well established in autism. GM volume is reported to be decreased at the left occipito-temporal junction in autistic children (Abell et al., 1999). Additionally, a post-mortem study on ASD brains found reduced neuronal density and volume in the fusiform gyrus bilaterally (van Kooten et al., 2008). Conclusively demonstrating that the disruption of the implicated areas occurred through the same connection between them or specifically excluding either short or long association fibres that might be found within the regions found would require individual tractography, which is beyond the scope of the current report.

In the right frontal lobe I found a reduction in GM volume at the right frontal pole and a reduction in WM volume in the right rostral cingulum. Functionally and structurally, the cingulum connects the medial frontal cortex to posterior cortices such as the posterior cingulate and precuneus as well as the thalamus and so is likely to be an important component of the mentalising circuit (van den Heuvel et al., 2008). Two VBM studies have also detected reductions in brain volume in ASD lateralized to the right; Abell et al. reported reduced GM volume in the right paracingulate cortex while Ke et al. found reduced WM volume in the right anterior cingulate (Abell et al., 1999, Neufang et al., 2008). When I compared male risk homozygotes to male heterozygote carriers and major allele homozygotes I observed a reduction in FA in the right rostral fronto-occipital fasciculus. This is the anterior portion of the same tract that I found reduced FA in proximal to the right fusiform. This is consistent with findings in DTI studies in autism where short range fibres have been found to have reduced FA within the frontal lobe in ASD patients as compared to controls (Sundaram et al., 2008). It is also suggested that the rostral prefrontal cortex, which encompasses it, may be affected in ASD as it has a more prolonged maturation time than most other brain areas (Huttenlocher, 1984, Dumontheil et al., 2008), which further suggests that frontal deficits may reflect either delayed growth in childhood or earlier pruning associated with maturation. CNTNAP2 has been shown by in situ hybridization and qPCR to be transcriptionally enriched within the anterior regions of the human brain (Abrahams et al., 2007), and regional expression may well explain specific findings in the rostral prefrontal cortex. Therefore CNTNAP2 transcripts from enriched prefrontal projection neurons translate Caspr2 could be expected to act axonally in the inferior fronto-occipital fasciculus in a trophic or developmental capacity on its projections in the occipital and other regions.

While the overall picture in the cerebrum suggested a deficit in a long-range circuit involving the frontal and occipital lobes and despite the existence of extensive fronto-cerebellar connections, in the cerebellum I observed only reductions in GM volume bilaterally and no differences in either white matter volume or FA. This is somewhat consonant with findings by Catani et al that FA is most reduced in short intracerebellar fibres in individuals with Asperger's and less so in long-range fibres (Catani et al., 2008). Specifically, I observed reduced GM volume in the posterior cerebellar hemispheres

bilaterally (crus I), the cerebellar vermis and the left superior cerebellum (lobule VI). Reductions in GM matter in several of these regions have already been implicated in ASD. In particular Rojas et al. found reduced GM volume in crus I of the posterior cerebellar hemispheres bilaterally in ASD, while Peterson et al. found reduced GM volume in the vermis in the non-autistic parents of autistic children (Rojas et al., 2006, Peterson et al., 2006). Indeed the cerebellum is the region of the brain where abnormalities have most frequently been found in ASD with MRI (Acosta and Pearl, 2004). One of the commonest ASD-associated finding is a reduction in number of cerebellar Purkinje cells and indeed the cerebellum has 70 billion out of the 85 billion neurons in the human brain so would be proportionately more vulnerable to intrinsic deficits in histiogenesis (Bauman and Kemper, 1985, Bailey et al., 1998, Williams and Herrup, 1988).The feedback loop between the cerebellum and frontal lobe involves intermediary projections to the thalamus and pons (Ramnani, 2006) and I did also find reductions in the white matter volume of the posterior thalamic radiation within the occipital lobes and reductions in the right anterior thalamic radiation only in females. The cerebellum has become increasingly implicated in higher cognitive processes and executive function such as those employed in theory of mind through these fronto-cerebellar circuits thus changes in cerebellum structure provide a potential anatomical basis for a range of cognitive deficits that are observed in ASD (Gordon, 2007). The cerebellum also has a role in language ability with structure associated with verbal fluency and word finding, and this could in part explain the previous association between CNTNAP2 risk polymorphisms and language impairments (Alarcón et al., 2008, Vernes et al., 2008), (Stanfield et al., 2008).

In conclusion, I discover reductions in GM volume, WM volume and FA in the fronto-occipital circuit and GM volume reductions in the cerebellum in healthy individuals homozygous for the risk allele of the CNTNAP2 rs7794745 polymorphism. I suggest a potential mechanism involving loss of a developmental or organizational function of Caspr2 acting in the axons of frontal projection neurons, with right lateralization, that has downstream effects through the fronto-occipital circuit or cerebro-cerebellar circuitry. However the mechanism requires further molecular genetic and cellular validation of the specific functional change induced by the risk allele and its effect in these circuits.

My broad approach in this study was to determine regions that varied with genotype and to implicate possible mechanisms accounting for the observed combination of changes in grey matter volume, white matter volume and FA. That the study focused on the more subtle genetic variance in normal controls allowed us to make the assumption that the identified tracts had the same connections identified in previous tractography studies. Nonetheless ongoing work with tractography and anatomical covariance will likely address further questions about network variability and properties raised in this study. While not appropriate for specific regional inferences, multivariate approaches such as those using kernels or latent variables could have useful applications in the fusion of these modalities and the use of collinearity between regions for development of predictive markers (Friston et al., 2008, McIntosh et al., 1996, Kloppel et al., 2009). Finally, my results suggest a strategy for testing of relevant cognitive processes and brain-based markers within genetically-characterised ASD populations.

4.5 Summary

Overall, I have found a number of sexually dimorphic phenotypes that are modulated by sex hormone-related polymorphism and that these same circuits modulated by sex hormones also appear to be affected by a recessive mutation of CNTNAP2 that confers risk for autism in males.

The structural covariance of amygdala volume with the posterior inferior temporal gyrus is modulated by AR CAG genotype. In females, the positive covariance of the left posterior inferior temporal gyrus was positively correlated with AR CAG, while in males the negative covariance of the right posterior inferior temporal gyrus was negatively correlated. The posterior inferior temporal gyrus also has larger GM and WM volume and increased FA in males and is modulated directly in GM and WM by AR CAG, ESR1 TA and ESR2 CA repeats. It is thought that the amygdalae are connected interhemispherically by the anterior commissure, which also communicates with other areas in the temporal lobe and I found that it was larger in females than males. However the anterior commissure was not associated with any of the polymorphisms investigated. Overall, the picture suggests that

the androgen receptor may act on a trophic connection to the temporal lobe perhaps via action on its structural connections or on the regulation of growth by other regions.

In females, the amygdala covariance of the left deep cerebellar nuclei or anterior cerebellar tonsil was modulated by ESR1 TA, and this is a region that is larger in GM and WM and has increased FA in females as compared to males. On the other hand, GM volume in the posterior lobe of the cerebellum as with GM and WM volume of the rostral prefrontal was associated with AR CAG, ESR2 CA and CYP19 TTTA repeats as well as with CNTNAP2 genotype. As mentioned, it has been suggested that sex hormones play a role in autism and the implicated circuitry here might point to a neural substrates where sex hormone pathways interact with pathways downstream of autism genes to influence development.

In this chapter, I have only investigated voxel-based differences as a basis for comparison between sex and genetic polymorphism. This renders inference of white matter differences somewhat more difficult as I have had to draw on existing knowledge about anatomical connections of the white matter tracts that appear to be involved. Many of these regions such as at the genu of the corpus callosum and the internal capsule contain crossings or contain axons joining very disparate regions that run together. Ongoing analysis with tractography will help to resolve these issues, but this is outside of the scope of this thesis. While investigating neural traits provides insight into the interactions and nature of the influence of sex hormone pathways on the brain, the important question is how this then relates to behavior and individual differences in personality.

4.6 Relevant conference presentations and publications

Christian Lambert, Geoffrey Tan, John Ashburner, Richard Frackowiak. Sex matters: A genetic basis for gender asymmetries in amygdala emotive networks. Organisation for Human Brain Mapping 2009.

Geoffrey C.Y. Tan, Thomas F. Doke, John Ashburner, Nicholas W. Wood, Richard S.J. Frackowiak. Variation in fronto-occipital circuitry, cerebellum and social hostility with autism-linked CNTNAP2. Organisation for human brain mapping 2010, Barcelona, Spain.

Geoffrey C.Y. Tan, Thomas F. Doke, John Ashburner, Nicholas W. Wood, Richard S.J. Frackowiak. Normal variation in fronto-occipital circuitry and cerebellar structure with an autism-associated polymorphism of CNTNAP2. NeuroImage 53 (2010) 1030–1042.

CHAPTER 5

PERSONALITY: NEURAL AND GENETIC CORRELATES OF SEXUAL DIMORPHISM IN GENDER ROLE ORIENTATION, NEUROTICISM AND EMPATHY

5.1 Abstract

This chapter considers the interplay of neural and genetic factors in the genesis of personality traits. In the first section, gender role orientation as defined by the Bem Sex Role Inventory is explored in terms of its correlations with sex hormone-related gene polymorphisms as well as 2D:4D. In the second section, I apply factor analysis to form orthogonal constructs of neuroticism across theoretically disparate measures. These factors are then investigated in terms of sex hormone-related gene polymorphism as well as 5-HTTLPR genotype and analysed for an influence on regional grey matter volume. In the third section, I relate these two sets of personality traits to social interaction and empathy first by a consideration of their covariance structure, then by association with gene polymorphism and brain structure.

5.2 Gender role

5.2.1 Introduction

The concepts of masculinity and femininity are essential pillars of the gender debate. They can be intensely polarising-on one hand oft discounted as completely social constructs by advocates of gender equality, on the other misused to further reinforce certain gender roles (Oakley, 1985). One of the ways of conceptualizing masculinity-femininity [M-F] has been to associate masculinity with dominance or instrumentality and femininity with nurturance or expressiveness. Thus masculinity includes traits such as assuredness and cold-heartedness, while femininity includes traits such as warmth, agreeableness and submissiveness (Bem, 1981). Another has been in terms of vocational preference within professions such as social work and teachings considered feminine and carpentry considered masculine (Eagly, 1987). The Bem Sex Role Inventory was designed in 1974 in order to measure masculinity, femininity and androgyny, the sum of positive masculine and feminine traits. Bem originally sought to assess 'the prevailing definitions of masculinity and femininity in the culture at large' a motivation grounded in the gender schema theory that sex-typing is derived from the readiness of an individual to encode and organise information in terms of the cultural definitions of maleness and femaleness constituting the society's gender schema (Bem, 1984, Bem, 1981). This self-concept in turn underlies sexually dimorphic cognitive processing and motivational dynamics. In the development of the inventory, a large number of men and women rated about 400 adjectives on how much more desirable for a man than a women and how much more desirable for a women than a man they were. They also rated a social desirability scale of how desirable an item is regardless of gender. From these adjectives, 20 masculine, 20 feminine and 20 neutral or socially desirable items were chosen (Wilson, 1999).

While the inventory claims to measure a societal or cultural construct, the method of self-report implies that in fact it derives from the individual or self-concept of participants. While the common assumption would be that this is still derived to primarily from gender

role socialisation where boys are differently reinforced as compared to girls, the confluence of recent evidence suggests the contrary. Sexual identity is primarily determined and assigned by the external appearance of the primary genitalia and in most gross respects gender role identity follows. However, there are numerous case studies in individuals with disorders of intersex where biological factors of sex appear to grate against socially formed gender roles. One such study describes a patient with 17 beta-hydroxysteroid dehydrogenase 3 deficiency was born with female genitalia and grew up as a female, but was genetically male and with age exhibited increasingly masculine behaviour and dysfunction. On being told she was genetically male, she adopted the male role and subsequently adjusted well as a man (Csathó et al., 2003, Lippa, 2006). Thus it would be reasonable to expect that normal variation in gender role identity could be influenced to a lesser extent by more subtle genetic polymorphisms of similar pathways.

I therefore sought to determine whether polymorphism in sex hormone-related genes could account for individual differences in gender role orientation.

5.2.2 Methods

To test this hypothesis, I first determined the range of sexually dimorphic and non-sexually dimorphic 'control' traits available from the subscales of the inventory. The author suggested 2 methods of scoring the questionnaire. The first, a median split method, divides the participants according to those scoring higher or lower than the median for masculine and feminine scores. This produces 4 groups which she describes as male or female sex-typed, androgenous and undifferentiated. The second involves subtracting feminine scores from masculine scores to produce a bipolar scale of masculinity/femininity. I also produce an additional score dividing masculinity by femininity in order to bypass the issue of social desirability. As my main interest lay in determining sexually dimorphic traits, the gold standard lay in the extent to which a given score was found to be differentiated between sexes.

I then performed partial correlations on SPSS controlling for sex between sexually dimorphic traits and polymorphisms of AR(CAG)n, ESR1(TA)n, ESR2(CA)n, CYP19(TTTA)n, PROGINS and MAOA VNTR as well as 2D:4D. As there was a specific hypothesis about the association of each polymorphism with each measure, correction for multiple comparisons was only performed post-hoc and a '*' is placed over associations that survive Bonferroni correction. Positive associations were further investigated to determine if they were driven by an effect in one sex or the other, and tested with a general linear model to detect sex interactions.

Recruitment, testing, imaging and genotyping were performed as previously described in the methods chapter.

5.2.3 Results

Sex role orientation was significantly different between males and females. Males scored significantly higher in masculinity (p=0.0014) and masculinity/femininity (p=0.00069), while females scored significantly higher in femininity (p=0.049). None of the other traits appeared to demonstrate sexual dimorphism.

Using partial correlations controlling for the effect of sex, M/F ratio was negatively associated with AR(CAG)n (-0.276, p=0.007*) and positively associated with the presence of the low expressing allele of MAOA (0.235, p=0.018). I further sought to determine whether this association was stronger in one sex and to detect sex-specific effects. The association with AR(CAG)n and MAOA appeared to be driven primarily by their correlation with M/F in females. M/F in females was negatively associated with AR(CAG)n (-0.208, p=0.036), the presence of the low-expressing allele in MAOA (0.366, p=0.002*) as well as carrier status for the PROGINS allele (0.224, p=0.019). In turn, the

associations M/F ratio in females appeared to be accounted for by masculinity scores with AR(CAG)n (-0.248, p=0.015) and PROGINS (0.340, p=0.001)*. On the other hand, MAOA low-expressing allele was both positively correlated with masculinity in females (0.291, p=0.011) and showed a trend towards negatively correlation with femininity in females (-0.206, p=0.054).

In males, M/F was not associated with either AR(CAG)n or MAOA, but instead was weakly positively correlated with polymorphism of the CYP19 gene (0.223, p=0.041) and negatively correlated with ESR1(TA)n (-0.208, p=0.050). None of these associations were found across sex or were significantly correlated with masculinity or femininity.

Using a general linear model to estimate an interaction of sex by factor, M/F ratio was showed a significant sex interaction with 2D:4D (F=10.535, p=0.002*), with M/F negatively associated with 2D:4D in females and positively associated with 2D:4D in males. M/F ratio was not significantly associated with the volume of any specific brain regions when performing voxel-based morphometry.

5.2.4 Discussion

The primary finding in this study that genetic variation significantly accounts for individual differences in sex role orientation partially refutes the originally held beliefs in the field that this personality trait is wholly cultural. Given that sex role orientation has been previously associated with 2D:4D(Bern, 1981), is strongly sexual dimorphic and intrinsically relates to how I define gender, the polymorphisms of genes for sex hormone receptors and CYP19(TTTA)n were ideal candidates. Indeed, none of the polymorphisms were associated to any degree with the neutral or social desirability scale and strongest associations were found with the 'bipolar' measure of M/F rather than any of the individual scales first developed by Bem under the gender schema theory (Hahn and Blakely, 2002). It thus seems unlikely that these findings could be attributed to chance.

Across sex, the AR(CAG)n and MAOA promoter VNTR polymorphisms were significantly associated with M/F. Increased expression of AR associated with decreasing (CAG)n length was associated with increased M/F. This was especially the case with trait masculinity in females where it appears that androgen receptor expression confers more male-like qualities. On the other hand carrying the low-expressing allele of MAOA, also called the warrior gene, was associated with increased masculinity and decreased femininity especially in women. This polymorphism of MAOA has been associated with traits such as response to social rejection, aggression and social conduct disorder in boys (Meyer-Lindenberg et al., 2006, Buckholtz and Meyer-Lindenberg, 2008). Additionally, it modulates the volume of structures such as the orbitofrontal cortex and amygdala (Sebastian et al.), and the neural processing of social rejection (Cattell and Scheier, 1961, Furnham, 1984). More commonly, its influences on brain and behaviour have been shown to be male-specific. Thus the finding that MAOA influences personality in females is relatively novel.

Despite the existence of multiple genetic associations with the BSRI, there were no regional differences in brain volume associated with the BSRI-derived measures. This remained the case when testing for sex-specific effects as well as for a sex interaction with these measures. This is somewhat surprising considering the extensive influences the associated genes appear to have on brain structure and at the same time somewhat suggestive as to the range of mechanisms by which they might influence masculinity and femininity. Where sex hormones and genetic differences in receptor expression and function may influence brain organisation during development and puberty, this would appear not to underlie individual differences in the genesis of gender roles. Instead, these traits may be linked to functional differences or responsiveness of relevant regions to sex hormones. Alternatively, it may have a more complex or subtle neural phenotype involving circuits or differences in connectivity that were not explicitly tested for in this analysis.

While gender orientation has direct relevance to the functional and social role of sexual dimorphism in personality, it is thought to account for a relatively small proportion of the individual variation underlying personality. I therefore decided to investigate sexual dimorphism in neuroticism, a major component of the big five factor model of personality with relevance to mood disorders.

5.3 Neuroticism

5.3.1 Introduction

In this chapter, I specifically investigate sex differences in the neural and genetic correlates of trait neuroticism. Neuroticism has been defined as a broad dimension of normal personality characterised by a tendency to experience chronic negative emotional states such as anxiety, sadness, anger or hostility and to display related behavioural and cognitive characteristics (Bolger and Schilling, 1991). Highly neurotic individuals generally have negative views of themselves and the world regardless of the objective reality. Thus individuals who score low on neuroticism appear to more stable and calm, and generally describe themselves as more happy and satisfied with their lives. It has been shown to be an important influence on stress vulnerability, where individuals with high neuroticism experience greater emotional distress from stressors such as overload at home or work and arguments (Cemalcilar et al., 2003). This has been shown to relate to both increased exposure to minor stressors as well as increased reactivity to stressful situations. Additionally high neuroticism is broadly associated with the tendency to exhibit 'learned helplessness' where negative situations and frustrations can be amplified to a state where the individual feels trapped (Saulsman and Page, 2004, Saklofske et al., 1995) and is considered to be a risk factor for a number of diseases including clinical manifestations of anxiety and depression(Feingold, 1994).

Neuroticism is one of the most consistent and replicated personality differences between men and women (Costa et al., 2001). While it is broadly discussed in popular psychology, there are also a number of studies over the years that provide consistent evidence for this. Feingold in his meta-analysis conducted in 1994 found that males score higher on measures of assertiveness, whereas females consider themselves to be more anxious, trusting and tenderminded (Flint, 2004). A later meta-analysis by Costa et al considering a broader range of traits in a number of cultures report higher negative affect, submissiveness and nurturance in women, and higher dominance in men(Lesch et al., 1996, Du et al., 2000). Thus females appear to score higher overall in aspects of neuroticism involving experience of negative affect and anxiety, and probably less well in aspects involving greater dominance. However the extent to which these differences have arisen from hormonal or biological differences as opposed to environmental or societal influences of the gender role remains unclear.

Neuroticism has been shown to have both a narrow, modeling only additive gene effects, and broad, incorporating non-additive components, heritability of 36-51% (Munafo et al., 2004). This suggests a significant genetic influence and validates the genetic approach. Additionally a number of gene candidates for neuroticism have been identified in recent years. Several studies in the past have found an association of the 5-HTTLPR polymorphism with neuroticism (Weiss et al., 2005). A genome-wide study on discordant twins suggests distinct genetic influences between males and females in addition to sex-independent associations (Hrdina, 2000). Furthermore large twin studies on neuroticism suggest that there is a greater relative contribution of non-additive genetic influences in males that appear to arise from epistatic interactions of additive gene effects. Yet, no study to date has specifically attempted to investigate the sex-dependent component of neuroticism or to understand the biological factors that may account for it.

Due to its clinical significance and associations with other measures, neuroticism has been one of the early focuses of psychiatric geneticists. A number of neural systems have been specifically implicated in neuroticism, particularly serotonin and the limbic system. For example, the serotonin transporter length polymorphism was found to be associated with

neuroticism in a healthy population by Lesch et al and this was found to be replicated in males but not in females by Du (Lee et al., 2005) but Munafo et al found no sex differences in this association(Wacker et al., 2005). The genetic architecture underlying blood serotonin levels, which appear to be mostly genetically determined, also appears to be different between sexes with a higher additive genetic component in females and loci at the serotonin transporter gene and ITGB3 appearing to influence serotonin levels only in males(Ebstein, 2006). Oestrogen has been shown to regulate regional expression of the serotonin transporter in the brain. Serotoninergic neurotransmission has been demonstrated to be different between males and females and has also been implicated in the depression through pharmacological studies and interventions. For example, levels of the serotonin metabolite 5-hydroxyindoleacetic acid and serotonin CSF and blood respectively are higher in women. PET studies have found higher global brain serotonin synthesis in men than women and lower regional binding capacity for an agonist for serotonin receptor 2 in the frontal and cingulate cortices of women. Women also tend to display a different pharmokinetic profile of serotonergic drugs, with lower plasma levels reached and shorter half-lives of tricyclic anti-depressants in men (Morissette and Di Paolo, 1993).

In addition to the more established links with serotonin, neuroticism also appears to be influenced by dopaminergic influences. Striatal dopamine receptor 2 density has been found to be higher in individuals with high neuroticism than those with low neuroticism (Rothbart et al., 2000, Rothbart and Derryberry, 1981). Additionally the TaqA1 polymorphism of DRD that influences striatal dopamine receptor density has also been shown to be associated with neuroticism and anxiety in men (Rothbart et al., 1994), while a 21 bp deletion causing a null mutation in the first exon of DRD4 has been identified as the causative mutation for OCD and panic disorder in a single individual (Carver and White, 1994, Jorm et al., 1998). Gonadal hormones are also known to influence neurochemical indices of dopamine function within the striatum, with ovarian hormone levels over the course of the estrous cycle underlying changes in striatal dopamine metabolism (Spielberger et al., 1970).

It is as yet unclear the extent to which trait neuroticism mediates the increased risk for depression in the female sex, although analysis of relatively large cohorts suggest that there is both an independent component of sex as well as a component mediated by personality. Identifying specific biological factors that may account for the sex-related component of neuroticism and understanding personality-related components in terms of their genetic and neural correlates. To do this, it is first necessary to understand how neuroticism is conceptualized across theories of personality in order to derive their psychometric constructs.

5.3.2 Methods

Trait neuroticism has been most frequently investigated in psychology by factor and correlational analysis of questionnaires. As discussed previously, it was first described as a construct of personality by a cross-sectional analysis of individual responses to trait-related word descriptions. Since then, a number of theories have arisen to posit possible mechanisms for individual differences in neuroticism-related behaviours.

The lexical approach of performing factor analysis of trait names from a dictionary has typically been shown to give rise to five broad factors (Norman 1963, Borgatta 1964, Digman and Takemoto-Chock 1981). The lexical hypothesis is based on the belief that most features of personality have become encoded in natural language such that the descriptions of personality within dictionaries provide a comprehensive set of attributes useful in daily interactions (Allport 1937). The big five inventory was developed on the basis of the adjectives defining these five factors with additional elaboration to improve clarity and consistency. Within this questionnaire, the authors describe neuroticism as contrasting ‘emotional stability and even-temperedness with negative emotionality, such as feeling anxious, nervous, sad and tense’ (John and Srivastava 1999).

The NEO-PI-R is perhaps the most well-validated and popular questionnaire currently used in personality research. It is frequently compared with new traits and questionnaires that are developed because of its good psychometric properties and has been the main flag-bearer for the five-factor model in recent years. In designing the NEO-PI-R, Costa and McCrae organised the five main domains of neuroticism, extraversion, agreeableness, conscientiousness and openness to experience hierarchically through facet analysis. Originally consisting of 3 domains, agreeableness and conscientiousness were added in light of evidence from other studies. The NEO was specifically guided by the five factor model, with an additional attempt to construct the domains conceptually from a number of facets based on second order factor analysis. Neuroticism was constructed from questions representing the facets of anxiety, angry hostility, depression, self-consciousness, impulsiveness and vulnerability(Schalling and Edman, 1993, Ekselius et al., 1994).

Temperament generally refers to stable individual differences that appear from birth and are believed to have a strong genetic and neurobiological basis. Most traits in temperament are derived from studies of individual differences in children. For example Rothbart and Derryberry, who define temperament as 'individual differences in reactivity and self-regulation assumed to have a constitutional basis', have developed an adult questionnaire on the basis of studies of enduring traits in infants and children, which contains major scales for aggressive and non-aggressive negative affect encompassing neuroticism(Francis, 1993). These include subscales such as fear and anger that have been related to the psychobiological models proposed by Gray(Stålenheim, 1997).

Grey suggests that neuroticism-related traits have their basis in animal behaviour where he describes five brain systems of 'arousal, reward, behavioural inhibition, consummatory response and fight/flight'. Behavioural inhibition can be thought of as controlling action through fear of negative consequences. Individuals exhibiting greater behavioural inhibition would have more anxious personalities due to their sensitivity to threatening signals such as for punishment, non-reward, novelty or with intrinsic fear properties. Approach behaviour on the other hand is controlled by the behavioural activation system that causes the organism to approach beneficial stimuli and has relations to impulsiveness. Thus these two

aspects of neuroticism are described as having differing biological basis and the balance between behavioural inhibition and activation determines whether an individual exhibiting increased neuroticism would be more anxious or impulsive in their behaviour(Gustavsson et al., 2000).

The State-Trait Anxiety Inventory is a popular and long-standing measure of anxiety and has been developed to measure anxiety specifically and not depression, which has been shown to be correlated with anxiety. Here I only measured trait anxiety, shown to be reliable and stable when retesting months to years later (Inc, 1985).

The State-Trait Anger Expression Inventory measures anger and hostility. These are components of the type-A behaviour associated with cardiac disease and overall aggression. The STAXI attempts to measure not only state and trait anger, but also its mode and intensity of expression such as how often anger is expressed towards other people or objects, suppressed despite being felt, or controlled. A study investigating the factor structure of STAXI discovered gender differences with men scoring more highly in factors representing state anger, trait anger and control, while women scored more highly in anger suppression suggesting that in addition to overall increased experience of anger as an emotion, men have different means of coping with it from women (Glass and Hopkins, 1996).

In the past few decades, more studies have tried to investigate the relationship between personality and health outcomes. The Karolinska Scales of Personality was constructed for research purposes based on hypotheses of biologically relevant disposition for vulnerability to psychological deviance and used by several researchers in psychological medicine (Kravitz et al., 2006). Comparison to earlier established questionnaires demonstrated that the domain of neuroticism as described by Eysenck could be made up of the KSP scales of psychic anxiety, somatic anxiety, muscle tension and psychasthenia while aggressiveness, an aspect of neuroticism in other questionnaires, related to inhibition of aggression, suspicion, guilt, indirect aggression, irritability, and verbal aggression (Dalla et al., 2004).

Among these scales, genetic factors have been shown to relate to neuroticism-related factors as well as aggressiveness scales of angry aggression and irritability. The instrument has been shown to measure traits that are stable after 9 years both in their absolute stability as well as relative or differential stability with neuroticism-related personality traits relating to both satisfaction with health in patients as well as functioning in family life and work (Nelson and Chiavegatto, 2001). It was subsequently updated to the Swedish universities Scales of Personality that was used in this study (Spampinato et al., 2009, Yamasue et al., 2007).

It is evident that one of the major differences between the models relates to the inclusion of aggression–related traits, which are sometimes grouped with agreeableness or extraversion. More recently, many researchers have defined neuroticism in terms of sadness and anxiety-related traits and investigate aggression independently despite factor solutions often placing them together. In this chapter, I include both in my definition of neuroticism although each is investigated separately after factor analysis. Overall, the factor-based models have been influential in defining the main constructs of personality and this is no less the case in neuroticism. While yielding statistical advantages by avoiding confounding measures or redundancy and providing a common framework within the field, such a model might be less useful clinically than one defining traits in terms of health outcomes or risk, in therapy for framing or developing interventions based on cognitive processes or in research than some of the psychobiological models. In this chapter, I also employ an approach, which can be similarly employed in these other realms, extracting such measures with respect to my specific biological elements of interest.

Recruitment, testing, genetics and imaging were performed as described in the methods chapter.

Statistical analysis was performed on SPSS (Ferrari et al., 2008). Principal component analysis was first performed to factor out correlated measures of neuroticism using their Pearson (product moment) correlations and derive orthogonal measures for subsequent

analysis. These were first tested for an association with genetic polymorphism using correlations. In most cases variables showed a normal distribution and Pearson correlation coefficients were used. Where tests for normality did not hold, Spearman rho coefficients were used. Point biserial correlations were used for dichotomous variables instead of two sample t-tests, although they have been shown to be mathematically equivalent to both Pearson product moment in coefficient and to the two sample t-test in the calculation of p-values (Frodl et al., 2008). A second order factor analysis was then performed on all variables with weighting in the first factor as the first factor appeared to weight most of the sadness and anxiety related variables. VBM analysis was performed as described in Chapter 3.2 on grey matter VBM including the orthogonal factors from the first order factor analysis in the design matrix. A separate VBM analysis was performed for the second order factor analysis. GM density across individuals was then extracted as eigenvariates from significant clusters in the second order analysis to test for an association with genetic polymorphisms in order to understand which neural substrates might mediate the associations. Finally sex and serotonin genotype were used to stratify the sample to determine the nature of the sex interaction with serotonin genotype on neuroticism.

5.3.3 Results

5.3.3.1 Factor Analysis

Here I derive a few broad composite measures of neuroticism from various constructs founded on different theories to find significant genetic and neural correlates of the trait.

To do this I first performed Principal Component Analysis [PCA] of traits that would be broadly considered to be related to neuroticism.

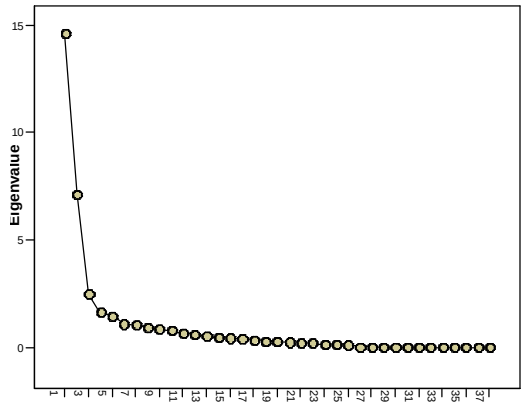


Figure 5.1 Scree plot of factors from PCA of neuroticism-related measures showing proportion of variance accounted for by each factor.

Initial factor analysis of all traits relating to negative emotionality including aggression demonstrated 6 factors of eigenvalue greater than 1.

	Factor weightings					
	1	**2**	**3**	**4**	**5**	**6**
Aggressive negative affect	0.942					
Non-aggressive negative affect	0.937					
Behavioural inhibition system (Gray)	0.924					
Physical trait aggression (Swedish)	0.924					
Mistrust (Swedish)	0.916					
Psychic trait anxiety (Swedish)	0.911					
Trait Irritability (Swedish)	0.911					
Somatic Trait Anxiety (Swedish)	0.908					
Angry reaction (STAXI)	0.861					
Frustration (ATQ)	0.848					
Fear (ATQ)	0.848					
Sadness (ATQ)	0.814					
Social anger (ATQ)	0.810					
Trait anger (STAXI)	0.682					
Anger expression-I (STAXI)	0.636	0.304		0.603		
Control In + Out (STAXI)	0.631			0.610		
Negative expressivity (BEQ)	0.593	0.396	-0.322		-0.341	
Stress Susceptibility (Swedish)	-0.590		0.576			
HA2 (TPQ)		0.834				
Neuroticism (Big five)		-0.826				
HA1 (TPQ)		0.816				
Trait anxiety (STAI)		-0.795				
HA3 (TPQ)		0.780				
Neuroticism (NEO-PI)		-0.584	-0.331		0.423	
Embitterment (Swedish)		-0.536	-0.422			
HA3 (TPQ)	-0.389	0.529				
Verbal Trait Aggression (Swedish)	-0.489		0.674			
Anger Control-O (STAXI)	0.318		0.624			0.306
Anger Expression -O (STAXI)	0.358		0.621			0.308
Anger Control-I (STAXI)	0.283			-0.421		-0.339

Table 5. 1 Simplified component matrix showing weighting of each measure with each of the six factors. Weightings were based on the correlation coefficients between factors and individual measures and are displayed when their absolute value exceeds 0.25.

Factor 1, accounting for 39% of the total variance, weighted measures relating to negative affect in both aggression and non-aggressive negative affect, and showed a significant correlation with CYP19(TTTA)n (0.225, p=0.010)* as well as sex difference (-0.291, p=0.001)* with females having higher scores than males. The second and third factors were both negatively correlated with AR(CAG)n (Factor 2, -0.255, p=0.005) (Factor 3, -0.217, p=0.014) although there was no significant sex difference in either factor. Factor 2 appeared to negatively weight anxiety and non-aggressive constructs such as big five neuroticism and harm avoidance, while positively weighting harm avoidance. Factor 3 appeared to relate to the dominant expression of negative affect positively weighting verbal aggression and stress susceptibility, and negatively weighting negative expressivity. The remaining factors showed relatively lower weightings with only a few measures weighted above 0.3. Factor 4 appeared to relate to the tendency to suppress or internalise anger while not being able to calm or cool off that anger. Factor 5 appeared to positively weight NEO neuroticism and negatively weight negative expressivity suggesting suppression of negative emotions. Factor 6 appeared to involve weightings from the STAXI anger questionnaire.

A further second order factor analysis was then performed on the anxiety and sadness-related traits excluding those related to aggressiveness. The first factor showed heavier weighting of anxiety-related measures, accounting for 69% of the variance. This factor was also significantly associated with CYP19(TTTA)n (0.233, p=0.006) and sex. The second factor in this analysis included traits that specifically defined sadness and was significantly associated with overall brain volume.

5.3.3.2 Analysis of sex by genotype interactions of 5-HTTLPR with components of neuroticism

One of the most well-established genetic links to neuroticism lies in the serotonin transporter promoter polymorphism. As described in the introduction to this section, there has been variability in reports on the nature of the sex by genotype interaction of gene association studies. I sought to determine whether this could be explained by differences in construct used. I looked for a correlation between increasing copies of the short or S allele of 5-HTTLPR with each of the 6 factors from the first order factor analysis.

Increasing copies of the S allele of 5-HTTLPR were associated with factors 1 (0.169, p=0.044), 3 (-0.177, p=0.037) and 5 (0.163, p=0.050). None of these survived correction for multiple comparisons across factors. In females, the S allele was only associated with factor 5 (0.357, p=0.003)* and in males, it was only associated with factor 3 (0.398, p=0.003)*.

Having described how different constructs appear to be differentially associated with 5-HTTLPR genotype between sexes, I wanted to determine if the sex differences in neuroticism, which is a much more robust difference, was itself different between 5-HTTLPR genotype. As previously mentioned, females scored higher in factor 1 (-0.291, p=0.001)* and males scored higher in factor 6 (0.330, p=0.000)*. In LLs, males scored significantly higher in factor 5 (0.459, p=0.007)* with a trend in factor 3 (0.236, p=0.113). There was also a trend for higher scores in females of the LL group in factors 1 (-0.312) and 4 (-0.291). In SSs, males also scored higher in factor 6 (0.434, p=0.007)*, while females scored higher in factor 3 (-0.328, p=0.033), with no significant sex difference in factor 1 (-0.171, p=0.175).

Using Bonferroni correction the positive association with factor 5 in females (0.357, $p<0.003$) and negative association with factor 3 in males (-0.398, $p<0.003$) as well as sex

difference with factor 5 in long allele homozygotes (0.459, $p<0.007$) were significant. S-allele dose was associated with factors 1, 3 and 5 overall but did not survive correction. Thus it can be seen that 5-HTTLPR is associated with different components of neuroticism between males and females and this might explain the observed differences in sex-selective associations between studies. Thus it would appear that serotonin genotype did not modulate sexual dimorphism of factors 1 or 6, but that there was a cross-over interaction in sexual dimorphism of factor 3 with LL males scoring higher than LL females (trend), but SS females scoring higher than SS males. This was confirmed with a GLM.

5.3.3.3 VBM analysis of the components of neuroticism, first order analysis

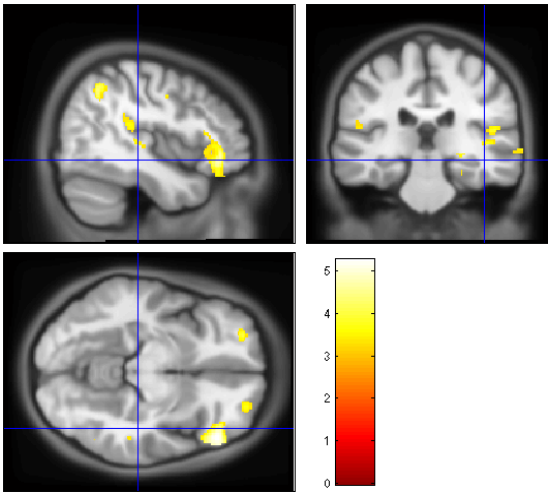


Figure 5.2 Regions showing significant association with Factor 1 of the first order analysis of neuroticism in GM volume. T-statistic maps thresholded at p>0.05 fwe-corrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

Factor 1 was significantly associated with volume of the right inferior frontal gyrus just anterolateral of the anterior insula and the right temporoparietal junction as well as the insula and rostral prefrontal bilaterally.

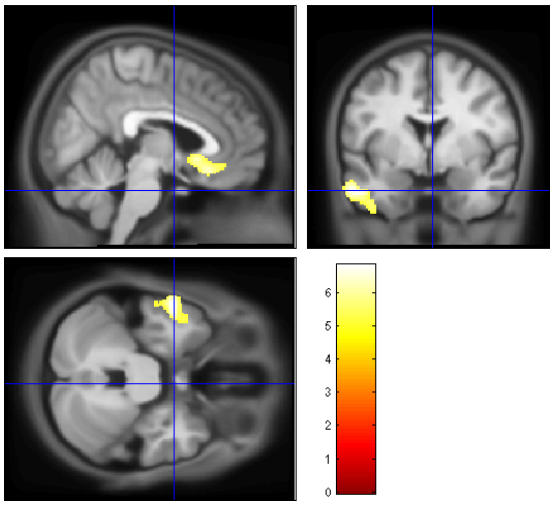


Figure 5.3 Regions showing significant association with Factor 2 of the first order analysis of neuroticism in GM volume. T-statistic maps thresholded at p>0.05 fwe-corrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

Factor 2 was significantly associated with the volume of the subgenual anterior cingulate cortex and left anterior temporal pole.

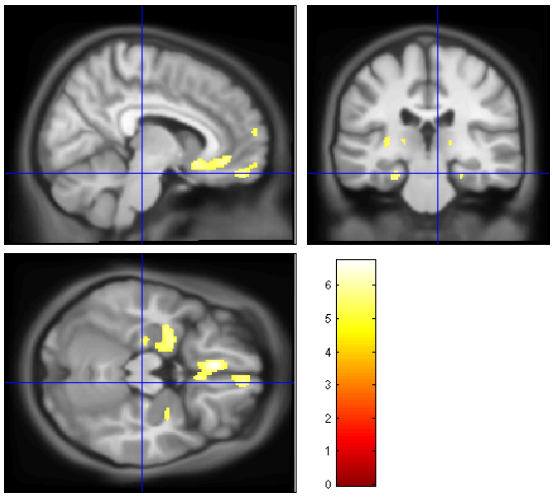


Figure 5.4 Regions showing significant association with Factor 3 of the first order analysis of neuroticism in GM volume. T-statistic maps thresholded at p>0.05 fwe-corrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

Factor 3 was negatively associated with the volume of the subgenual anterior cingulate cortex as well as medial orbitofrontal cortex, amygdala and parahippocampal gyri bilaterally.

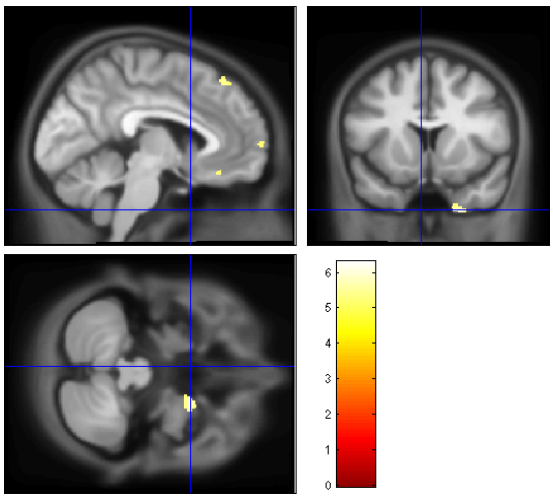


Figure 5.5 Regions showing significant association with Factor 4 of the first order analysis of neuroticism in GM volume. T-statistic maps thresholded at p>0.05 fwe-corrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

Factor 4 was also associated with volume of similar regions in the medial prefrontal cortex as well as the right anterior temporal pole near the amygdala.

Factors 5 and 6 had no significant grey matter correlates.

5.3.3.4 VBM Results of second order factor analysis

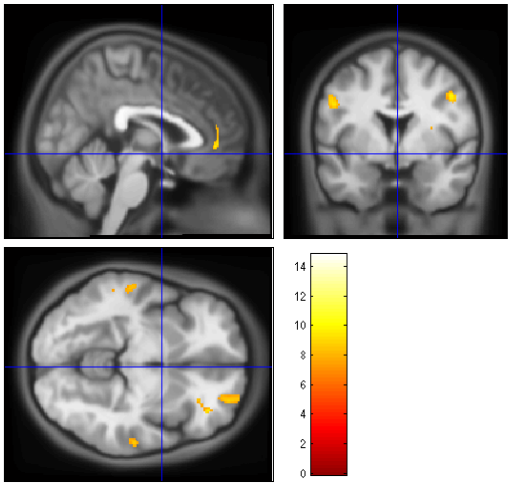


Figure 5.6 Regions showing significant association with Factor 1 of the second order analysis of neuroticism in GM volume. F-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

Factor 1 of the second order analysis was significantly associated with regions of the lateral temporal cortex and dorsolateral prefrontal cortex bilaterally as well as the anterior cingulate and left BA10.

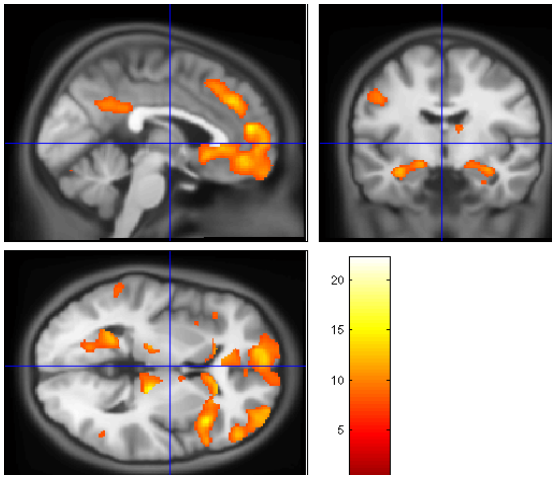


Figure 5. 7 Regions showing significant association with Factor 2 of the second order analysis of neuroticism in GM volume. F-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

Factor 2 of the second order analysis was significantly associated with the orbitofrontal cortex including BA10, dorsomedial prefrontal cortex, subgenual ACC, amygdala and hippocampus.

5.3.3.5 Mediation analysis of regions associated with neuroticism and genotype

As factor 1 of the first order analysis was be sexually dimorphic, I sought to determine whether the individual regions associated with the second order factor analysis were similarly associated with sex and genotype and if these could then account for sex and gene associations with personality.

Factor 1 was associated with sex, CYP19(TTTA)n genotype and 5HTTLPR genotype. Factor 2 was associated with PROGINS genotype and not with sex. Of the 9 regions significantly associated in grey matter volume with factor 1 independent of sex effects (p<0.001 uncorrected), 5 were significantly associated with sex- the right dorsolateral prefrontal cortex, right parietal cortex, left lateral temporal cortex, left dorsolateral prefrontal cortex and right frontal pole (BA10) with a trend in the left insula. None of the

regions associated with factor 2 were also associated with sex. The right frontal pole appeared to be associated in volume with both CYP19(TTTA)n and PROGINS genotypes in both sexes. PROGINS genotype was also associated with volume of the right orbitofrontal cortex. In regions associated with factor 2, CYP19(TTTA)n was also associated with volume in the right thalamus, and PROGINS exhibited a trend with left dorsomedial prefrontal cortex and left fusiform cortex.

5.3.4 Discussion

Aggressive and non-aggressive traits appeared to be grouped together in factor 1. This could be because of the wide spectrum of definitions of neuroticism such that different measures weighted aggressiveness to a varying degree. Aggression is known to be positively associated with neuroticism, but this association is modulated by agreeableness and extraversion (Sterzer and Stadler, 2009, Nelson and Trainor, 2007). Second order analysis of only non-aggressive traits yielded a clearer factor structure with, the first factor accounting for 69% of the variance as compared to 39% of the variance accounted by factor 1 of the first order analysis. Factor 2 of the first order analysis appeared to capture variance with anxiety-related traits, while factor 3 appeared to relate to dominance in expression of negative affect.

This is the first report of an association between CYP19 and neuroticism. There are as yet relatively few studies demonstrating a link between CYP19 and the brain or personality. Yet this is a strong candidate for a sexually dimorphic component of neuroticism. One study demonstrated an association of SNPs in CYP19 with depression in premenopausal women (Eastley and Wilcock, 1997). Female aromatase knockout mice show increased depressive-live behaviours(Nelson and Trainor, 2007). From the second order analysis, the factor associated with CYP19 and sex was more heavily weighted by anxiety, although anxiety and sadness-related traits were closely related.

The association of androgen and AR polymorphism with aggression-related traits found in factor 3 and decreasing anxiety on the other hand is relatively well-characterised. In domesticated mammals, castration has been traditionally employed to eliminate aggression while androgen replacement restores it. Additionally male mice with a mutation of the androgen receptor lack aggressiveness(Amodio and Frith, 2006, Adolphs, 2003). It may be that the component of aggression that is accounted for by androgen receptors relates more strongly to social dominance than aggressiveness in humans *per se*.

5-HTTLPR genotype appeared to be associated with different factors as well as common factors between men and women, with significant differences across sex in factors 1, 3 and 5, but specific associations with the neuroticism and negative expressivity-related factor 5 in females and with the social dominance-related factor 3 in males. 5-HTTLPR genotype has been broadly associated with several facets of neuroticism in normals and patients including aggression, anxiety, depression and serotonin has been implicated in these traits and behaviours in a wide range of animal models (Preston and de Waal, 2003). From this and other genetic studies investigating the sex interaction with serotonin genotype in neuroticism, it is clearly apparent that the same genotypic difference has distinct manifestations between males and females. While the S allele is broadly associated with increased neuroticism, it seems to relate more to increased anxiety in females and aggression in males. In turn, serotonergic background as indicated by 5-HTTLPR genotype influences the propensity for certain factors to exhibit sexual dimorphism. Thus my findings suggest that sex modulates factor 3 through largely through an influence on serotonergic transmission, while factors 1 and 6 exhibit sexual dimorphism via other mechanisms.

Anxiety in healthy volunteers(Eisenberg and Lennon, 1983, Loon, 2009) as well as in patients with generalized anxiety (Hoffman and Levine, 1976) has been associated with reductions in grey matter volume of the amygdala, hippocampus, temporal particularly the superior temporal gyrus and prefrontal cortices. Depression has been associated with longitudinal decline in grey matter in the hippocampus, anterior cingulum, amygdala, and dorsomedial prefrontal cortex(Olweus and Endresen, 1998). In concordance with these

studies, Factor 1 was associated with the right IFG and rostral prefrontal as well as the posterior aspect of the superior temporal gyrus at the temporoparietal junction. However it did not appear to be associated with the volume of the amygdala or hippocampus. Factors from the second order factor analysis, which excluded all of the aggression-related measures, were associated with the lateral temporal cortex, dorsolateral and dorsomedial prefrontal, anterior cingulate, BA10 and hippocampus, and thus captured more of the circuitry that has been implicated in anxiety and depression.

Subgenual anterior cingulate cortex, temporal poles, orbitofrontal and medial temporal cortex including the parahippocampal gyrus and amygdala were associated with factors 2 and 3, which had high weightings for aggression-related measures. Individuals with increased aggression or violent behaviour have been shown to have reduced volume or activity in the orbitofrontal cortex in emotional regulation, anterior cingulate cortex in cognitive control, amygdala in emotional recognition and temporo-parietal cortex in performance monitoring(Karniol et al., 1998). The temporal poles and parahippocampal gyrus have not been broadly implicated in disorders of aggression, although the parahippocampal gyrus has been associated with aggressive behavior in Alzheimer's disease(Watson et al., 1994) and the hippocampus is important in the mediation of aggressive behavior in animal models(Guarino et al., 2007). Grey matter volume in the medial prefrontal cortex and right anterior temporal pole were associated with factor 4, which was associated with people who scored lower on anxiety and sadness measures, while scoring higher on aggressiveness measures and might be interpreted as relating to social dominance. Indeed these regions have been typically associated with theory of mind and social cognition(De Vignemont and Singer, 2006).

In investigating the regions associated with the second order factor analysis, it appeared that prefrontal and temporoparietal cortices might mediate the sex differences found in factor 1. Of these the right frontal pole appeared to be associated with CYP19(TTTA)n and PROGINS genotype. Thus it may be that sex hormones influence factor 1 differently between sexes through an effect on BA10. Resting glucose metabolism in BA10 has been shown to be negatively correlated with neuroticism in BA10

Thus I describe the neural and genetic correlates of various components of neuroticism and through an analysis of their interactions attempt to explain their relationship to one another. In the next section, I investigate the link between these and traits relating to social interaction.

5.4 Empathy and social affiliativeness

5.4.1 Introduction

Gender-role orientation and emotional make-up are important in shaping a range of behaviours perhaps most importantly in the realm of social interaction. As eusocial organisms, humans gain an enormous survival advantage unavailable if I were to operate as individual agents (Nowak et al.). Thus it is of great interest to discover how social traits are wired to allow us to interact successfully with other humans, form relationships and organise ourselves in a way that fosters cooperation. Among the social traits, empathy is probably among the best characterised and most extensively studied.

Sex differences in empathy have been found across a number of studies. In general self-report scales show the largest differences favouring women, with moderately higher scores in women for reflexive crying and self-report under laboratory situations and no differences when using physiological reactions or their facial expressions and gestures when viewing an emotional response(Hoffman and Levine, 1976). In a meta-analysis by Hoffman, females score higher on what he terms as vicarious affective arousal where women are more likely to share emotional distress(Evans and Rothbart, 2007, Rothbart, 2007). In a study of children from early childhood to adolescence considering empathic responses to the emotional states of boys and girls, girls show increasing empathic concern towards both girls and boys with increasing age during childhood while boys show a similar

developmental pattern towards girls and a decreasing trend in empathic concern towards boys in distress. One study of empathy in adolescence found that the gender difference in empathy as measured by the interpersonal reactivity inventory could be explained by the femininity scale of the Bem gender-role orientation inventory discussed earlier, while being unrelated to the masculinity scale. Another study using masculinity and femininity scales in addition to those measuring narcissism from the personality attributes questionnaire found that healthy forms of empathy and communal orientation were associated with higher socially desirable femininity and lower socially undesirable masculinity.

Empathy has also been associated with negative affect and various components of neuroticism, although the multifaceted nature of neuroticism has resulted in inconsistency in this association. Using the interpersonal reactivity inventory to measure empathy, negative egocentric sensitivity on the emotional sensitivity scale, a strong surrogate marker of trait neuroticism, has been strongly positively correlated with personal distress and positively correlated with a general measure of emotional empathy, while being negatively correlated with perspective-taking. Positive interpersonal sensitivity on the other hand was strongly positively correlated with empathic concern, perspective taking and general emotional empathy, but is unrelated to neuroticism. Thus neuroticism appears to relate to components such as emotional distress and other more affective components of empathy via negative emotional sensitivity.

Empathy has been described as the act of constructing another person's mental state or the experience of an affective response more appropriate to another's situation than one's own and is a fundamental cognitive process underlying social interaction. De Vignemont states that in order for empathy to be present as a cognitive process, one must be in an affective state that is isomorphic to another person's affective state, elicited by imagination or observation of their affective state and one must know that the other person is the source of one's own affective state. Thus an empathic individual have traits conducive to meeting those criteria. De Waal describes three components from this psychometric perspective, namely emotional response to someone else and their emotional state, the cognitive ability to take the perspective of another person or theory of mind and the ability to regulate one's

own emotions and external response. The first component has its basis in shared affect or emotional contagion, a concept that originated in developmental psychology, where ones emotional state spontaneously mimics that of the other person and Hoffman has also termed this vicarious affective response. He termed the second as recognition of affect or affective perspective taking and defines it as the ability to recognise emotional state and intentions from bodily, nonverbal and situational cues.

In this section, I first describe the psychometric relationship between social traits such as social affiliativeness with components of neuroticism and sex role orientation in order to better understand define the constructs common to both and to pinpoint the sexually dimorphic components of social cognition and empathy. I expand this analysis to determine the genetic components of social affiliativeness and its subscales before looking at the grey matter correlates for this trait. By so doing, I seek to make inferences on the nature of sexual dimorphism in empathy.

5.4.2 Methods

Social affiliativeness has been defined as concern for others and has been proposed as a dimension of temperament in the adult temperament questionnaire. In children, affiliativeness is associated with greater tendency to exhibit internalising problems as opposed to externalising. It has been shown to be related to Big Five Agreeableness and differs from sociability in that the latter involves a preference for conversing, interacting and approaching others. It has two subscales: Emotional empathy, defined as the tendency to experience affective responses congruent with the feelings of others; and empathic guilt, experiencing distress in response to negatively affecting other people. A third subscale, known as social closeness, was found to have poor psychometric properties and consistency with overall affiliativeness so was excluded from the questionnaire.

Personality questionnaires were administered as described in the introduction to this chapter and measures of social traits including social affiliativeness on the adult temperament questionnaire were measured. I first determined whether empathy related to measures of neuroticism such as aggression and anxiety. I then looked for associations between measures of empathy and my genes of interest. Finally, I performed VBM as described in the methods chapter looking for regions where GM volume was significantly associated with social affiliativeness across sex, within sex and where there was a significant interaction with sex.

5.4.3 Results

5.4.3.1 Relationship to neuroticism

I first determined correlates of social and empathy-related traits with factors derived from the first order PCA of neuroticism. Social affiliativeness from the adult temperament questionnaire was positively associated with factor 1 (0.320, $p<0.001$)*, and negatively associated with factor 3 (-0.281, $p=0.002$)*, factor 4 (-0.238, $p=0.027$), factor 5 (-0.313, $p=0.001$)* and factor 6 (-0.237, $p=0.010$). Social affiliativeness has two subscales, emotional empathy and empathic guilt, which were similarly associated with neuroticism factors. Multi-dimensional empathy contains six scales, suffering, positive sharing, responsive crying, emotional attention, feeling for others, emotional contagion and general empathy. Suffering was negatively associated with factor 3 (-0.307, $p=0.003$)*, factor 4 (-0.234, $p=0.026$) and factor 6 (-0.257, $p=0.014$). Positive sharing was negatively associated with factor 4 (-0.234, $p=0.026$) and factor 6 (-0.369, $p<0.001$)*. Responsive crying was negatively associated with factor 6 (-0.403, $p<0.001$)* and positively associated with factor 1 (0.386, $p<0.001$)*. Emotional attention was negatively associated with factor 4 (-0.272, $p=0.009$), factor 5 (-0.256, $p=0.014$) and factor 6 (-0.357, $p<0.001$)*. Feeling for others was positively associated with factor 1 (0.282, $p=0.007$). Emotional contagion was not significantly associated with any neuroticism factors. General empathy was negatively associated with factor 3 (-0.222, $p=0.034$) and factor 6 (-0.403, $p<0.001$).

In terms of sex role orientation, masculinity was negatively associated with emotional guilt (-0.239, p=0.025). Femininity showed a positive, but non-significant trend with social affiliativeness (0.199), emotional empathy (0.205) and emotional guilt (0.149). M/F was negatively associated with social affiliativeness (-0.322, p<0.001), emotional empathy (-0.244, p=0.02) and empathic guilt (-0.326, p<0.001).

Social affiliativeness was also significantly greater in females than males both overall (p<0.001), and in its subscales of emotional empathy (p=0.012) and empathic guilt (p=0.001). Trait aggression has been previously found to be associated with the MAO VNTR and AR CAG genotype, while ESR1 TA and AR CAG genotypes modulate sexually dimorphic traits.

5.4.3.2 Gene associations with social traits

ESR1(TA)n was significantly negatively correlated with social affiliativeness (-0.144, p=0.041) with a nonsignificant trend with its subscales of emotional empathy and empathic guilt. The low expressing allele of MAO VNTR was positively associated with social anger (0.248, p=0.003) and aggression (0.214, 0.010) and negatively associated with emotional empathy (-0.170, p=0.034). AR(CAG)n showed no significant associations.

As autism is characterised by abnormalities in social interaction, I investigated whether CNTNAP2 genotype was associated with social traits such as empathy, social affiliativeness and autistic quotient. Risk homozygotes scored higher than carriers and non-risk homozygotes in Mistrust (mean diff.=1.99, sig. (1-tailed) p=0.019) and Anger responsiveness (mean diff.=1.33, sig. (1-tailed) p=0.027). Additionally female risk homozygotes scored higher than the other females in Autistic quotient (mean diff.=7.42, sig. (1-tailed) p=0.019) and Trait anger (mean diff.=1.81, sig. (1-tailed) p=0.012). No

significant differences by genotype were found in the male cohort or in autistic quotient and various measures of empathy across sex.

5.4.3.3 VBM with social affiliativeness and its subscales

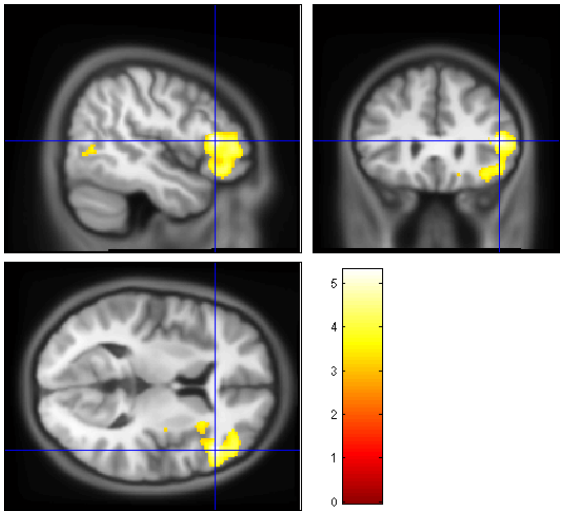


Figure 5.8 Regions positively associated with social affiliativeness of the ATQ in GM volume. T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

Social affiliativeness was positively correlated with GM volume in the right anterior insula and right lateral inferior and middle frontal gyri.

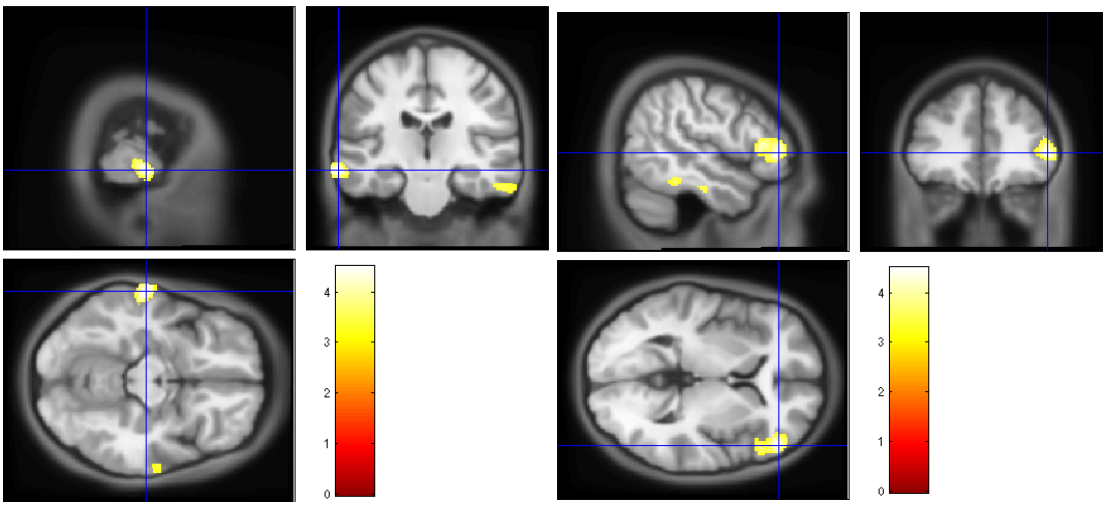


Figure 5.9 Regions positively associated with the emotional empathy subscale of social affiliativeness in GM volume. T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

Emotional empathy was positively correlated with GM volume in the lateral inferior temporal gyri bilaterally and the right lateral prefrontal cortex

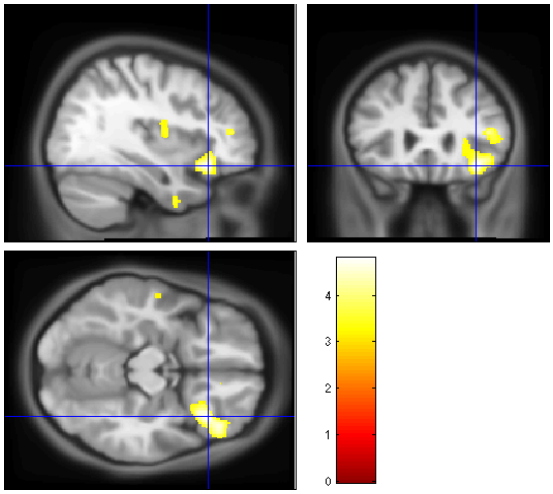


Figure 5.10 Regions positively associated with the empathic guilt subscale of social affiliativeness in GM volume. T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

Empathic guilt was positively correlated with GM volume in the right orbitofrontal cortex and right anterior insula.

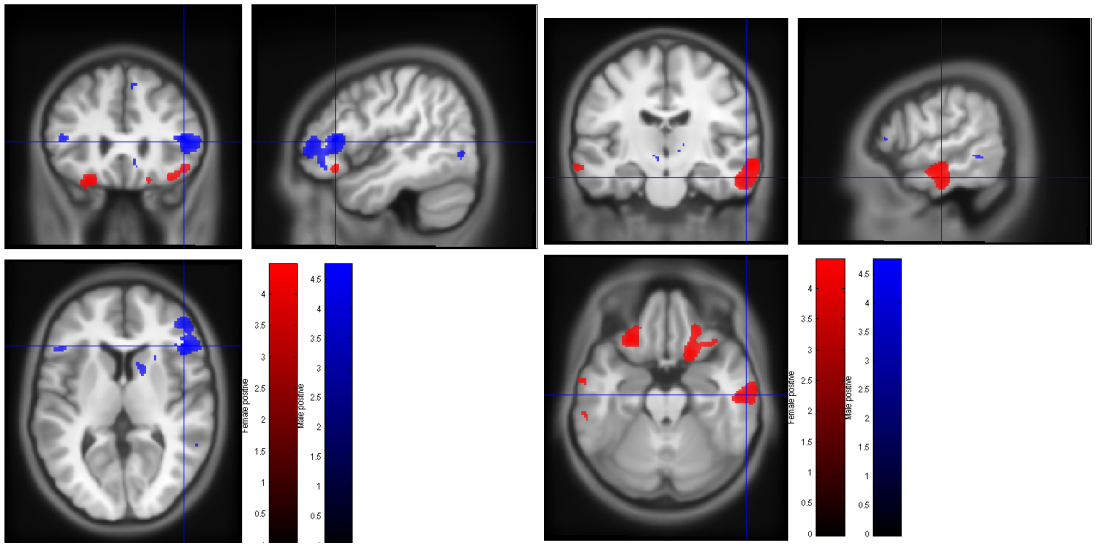


Figure 5.11 Male and female GM correlates with social affiliativeness. Positive associations of social affiliativeness with GM volume in males (blue) and females (Red). T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

Males and females appeared to have distinct neural correlates of affiliativeness. In females social affiliativeness was positively correlated with GM volume in the lateral temporal and orbitofrontal cortex, while in males it was positively correlated with GM volume in the right lateral prefrontal cortex.

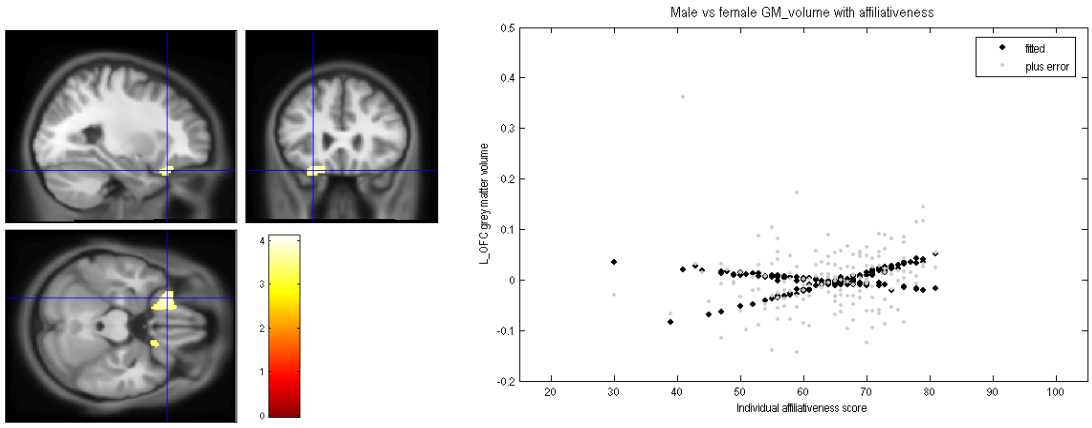


Figure 5. 12 Sex interaction with social affiliativeness in GM volume. Regions showing significantly greater increases in GM volume with increasing social affiliativeness in females than males. T-statistic maps thresholded at p>0.001 uncorrected were overlaid on the average of individual T1-weighted images warped together into MNI space.

Within the orbitofrontal cortex, females showed significantly greater GM volume increases with increasing social affiliativeness than did males.

5.4.4 Discussion

Social affiliativeness and its subscales as well as several scales of multi-dimensional empathy were associated with all factors of neuroticism except for factor 2, suggesting that most aspects of neuroticism seem to affect emotional empathy and empathic guilt. At first, this would seem to be at odds with inconsistency of neuroticism's association with empathy across various studies. However, the direction of the association between factors differed, with factors such as aggression related negatively to affiliativeness and non-aggressive affect related positively. Thus the nature of the psychometric construct and the inclusion or exclusion of aggression-related items would influence the association. Additionally, masculinity over femininity was negatively associated with social affiliativeness and its subscales, which is in agreement with other studies investigating sex role orientation with empathy.

The CNTNAP2 risk allele was associated with mistrust, and anger responsiveness. In females and not in males, the risk allele was associated with autistic quotient and trait anger. While these are more social components of aggressiveness and related to social interaction, they aren't directly related to empathy or theory of mind. It would be interesting to see if mistrust and anger responsiveness are also more common manifestation in risk homozygotes with autistic spectrum disorder. The female-specific association with CNTNAP2 was somewhat unexpected as the initial hypothesis was that the autism quotient and empathy-related traits would be associated with risk homozygosity in males. However it is in concordance with the finding that most of the brain-based differences were found in both sexes.

It appeared that disparate anatomical networks accounted for individual differences in trait affiliativeness between men and women. Where more socially affiliative men had greater GM volume in the right lateral prefrontal cortex, more socially affiliative women appeared to have larger inferolateral temporal and orbitofrontal cortices. It is notable that in my study, the inferior temporal and medial orbitofrontal cortices were larger in women, while

anterior insula and lateral inferior gyrus of the prefrontal cortex were larger in men and it may be that these sex differences provide differing substrates for the development of empathy, guilt and its more basic cognitive components. Of these regions, the orbitofrontal has been conventionally associated with social cognition via emotional regulation and motivation (Adolphs, 2003), the anterior insula is thought to be important in the experience of emotions such as pain and disgust, and the inferior frontal gyrus is commonly activated in response to action observation (Frith and Frith, 2007).

ESR1(TA)n and the low expressing allele of the MAOA VNTR were negatively correlated with social affiliativeness and empathy respectively. Thus it would appear that decreased expression of the oestrogen receptor is associated with increased social affiliativeness. I previously showed that ESR1(TA)n is also negatively associated with lateral temporal cortex GM volume, while I show here that lateral temporal GM volume is positively associated with social affiliativeness in females and emotional empathy across sex. Thus it may be that oestrogen receptor alpha acts within the inferolateral temporal lobe to influence the propensity for empathy. On the other hand, the low expressing allele of MAOA VNTR or the warrior gene is related to aggressiveness and social conduct disorder in children and has been shown to influence orbitofrontal cortex volume in men. Criminals and violent offenders have been shown to score lower on most measures of empathy so it would be interesting to investigate into whether aggression, violent behaviour and empathy have a common basis in the orbitofrontal action of monoamine oxidase and the neurotransmitters it metabolises.

5.5 Summary

In Section 5.1, I elucidated genetic influences underlying gender role orientation. M/F was negatively associated with AR(CAG)n and positively associated with the low expressing allele of MAOA VNTR. Additionally carrier status for the PROGINS allele and the low expressing MAO VNTR allele was negatively correlated with M/F only in females accounted for by masculinity scores. M/F also showed a sex interaction with 2D:4D, being positively associated with males and with a negative trend in females.

In Section 5.2, I attempted to understand the genetic and neural correlates of statistically separate factors of neuroticism. I used principal component analysis to determine orthogonal components of neuroticism from a number of commonly used measures. Six of the components had eigenvalues larger than one.

- Factor 1 Negative affect. (Majority of the joint variance across measures)
- Factor 2 Anxiety-related measures including harm avoidance, big five neuroticism and STAI anxiety.
- Factor 3 Mode of expression of negative affect with greater aggressive as opposed to non-aggressive expression.
- Factor 4 Increased aggressiveness with decreased non-aggressive negative affect.
- Factor 5 Neuroticism and negative expressivity.
- factor 6 Anger-related measures.

I discovered that factor 1 was associated with CYP19(TTTA)n and was sexually dimorphic, while factors 2 and 3 were associated with AR(CAG)n. 5-HTTLPR genotype showed a significant interaction with sex in neuroticism, with males and females demonstrating associations with 5-HTTLPR with different components of neuroticism, and the sex difference in neuroticism differing by genotype. The main neural substrates for neuroticism appeared to involve various regions of the temporal and prefrontal lobe

including the anterior cingulate gyrus with BA10 possibly mediating the association between CYP19(TTTA)n and factor 1.

In Section 5.3, I attempted to relate measures from section one and two to social traits and empathy. Social affiliativeness was positively correlated with factor 1 and negatively correlated with factors 3 and 5. Empathetic suffering was negatively correlated with factor 3. Positive sharing was negatively correlated with factor 6. Responsive crying was negatively correlated with factor 6 and positively with factor 1. Emotional attention was negatively correlated with factor 6. Feeling for others was positively correlated with factor 1. General empathy was negatively correlated with factors 3 and 6. Thus factors 1, 3 and 6 were most strongly correlated with aspects of empathy and affiliativeness. Individuals with greater negative affect as the first factor appeared to experience stronger affiliativeness expressed as guilt and empathy with stronger feeling for others and responsive crying, but not general empathy. Individuals with greater tendency to express negative emotions as aggressiveness regardless of overall negative affect were consistently less affiliative and scored lower in positive sharing and general empathy. Individuals with the tendency to internalise anger and negative emotions tended though less strongly significant to be less affiliative, experienced less empathetic suffering, positive sharing and were less emotionally attentive. Individuals with a greater tendency to express their aggression outwardly, but who were also bad at calming inner emotions by themselves, were also less likely to experience positive sharing, responsive crying, and were less emotionally attentive and empathic generally. Those who scored higher on masculinity were less likely to experience empathic guilt. While those scoring higher on M/F were less socially affiliative, emotionally empathic and scored lower on empathic guilt. ESR1(TA)n was negatively correlated with social affiliativeness, carrying the low expressing allele of the MAO VNTR was negatively correlated with emotional empathy, and homozygosity for the risk allele of CNTNAP2 was associated with higher mistrust and anger responsiveness, with higher autistic quotient and trait anger in females. Social affiliativeness also appeared to have distinct neural correlates between men and women and the suggestion that the lateral temporal cortex mediates the effect of ESR1(TA)n on social affiliativeness.

5.6 Relevant conference presentations and co-authored publications

Geoffrey CY Tan, Katie Owens, Jon Roiser, Catherine Sebastian, Carlton Chu, John Ashburner, Nicholas W Wood, Richard SJ Frackowiak. Neural and genetic bases for social affiliativeness and aggression. Oral presentation 717.7, Social Cognition: Perceiving Social Agents. Society for Neuroscience 2008, Washington DC, USA.

C. L. Sebastian, J. P. Roiser, **G. C. Y. Tan**, E. Viding , N. W. Wood , S.-J. Blakemore. Effects of age and MAOA genotype on the neural processing of social rejection. Genes, Brain and Behavior**.** Volume 9, Issue 6, pages 628–637, August 2010.

Jonathan P. Roiser, Benedetto de Martino, **Geoffrey C. Y. Tan**, Dharshan Kumaran, Ben Seymour, Nicholas W. Wood, and Raymond J. Dolan. A Genetically Mediated Bias in Decision Making Driven by Failure of Amygdala Control. The Journal of Neuroscience, May 6, 2009, 29(18):5985-5991.

Bryan A. Strange, Marijn C. W. Kroes, Jonathan P. Roiser, **Geoffrey C. Y. Tan**, and Raymond J. Dolan. Emotion-Induced Retrograde Amnesia Is Determined by a 5-HTT Genetic Polymorphism. The Journal of Neuroscience, July 9, 2008, 28(28):7036-7039

CHAPTER 6

DISCUSSION

6.1 Abstract

From its outset, this thesis has attempted to pose and address two basic questions: Can gene-based variation within and across sex provide insight into the molecular basis for sexual dimorphism in brain and behaviour? And, what insight can be gleaned into the possible mechanisms for sexually dimorphic disease risk, based on the neural and genetic correlates of disease-relevant factors and endophenotypes?

I first cover the theoretical considerations around my approach to answering these questions, in terms of methodological and inference-related limitations, as well as their significance for mechanistic follow-up studies and disease. I use these perspectives to guide further discussion of the implications of my specific findings in each domain: Whole brain volume and grey matter; anatomical networks in structural covariance and white matter; and aspects of personality such as gender orientation, neuroticism and empathy.

6.2 Theoretical issues

It is not possible in either question to claim that my findings provide a complete answer. In this section I discuss methodological issues pertaining to statistical validity, limitations of using genetics, limitations of using structural MRI and finally therapeutic relevance.

Firstly, while statistically significant, each finding would benefit from replication as this is but one study. Additionally, the study cohort is a relatively homogenous population of fairly well-educated young Caucasian adults of British origin. As such, the results may not generalise to dissimilar populations depending on the extent to which population differences modify the traits I have investigated. However the majority of findings appear to be corroborated by evidence from other studies. As yet there has been little to suggest that population differences would interact with the gene effect on these traits or that the mechanism of hormone action would be different in another population.

A genetics study has very poor temporal sensitivity in determining the stage of life at which a gene effect occurs. A gene polymorphism will modulate the activity or expression of its protein of interest consistently from embryogenesis until the end of life, so a study of genetic differences in adulthood should have the power to detect lasting differences produced at any stage. However, whether that difference occurred in the womb, in childhood, in puberty or early adulthood is unclear. With some of the results such as hippocampal and temporal lobe volume with AR(CAG)n, there was a clear genotype by age interaction, in which case it is possible to determine that the influence occurs over the age range tested. But where this is absent, further investigation in the womb, childhood and post-puberty both cross-sectionally and longitudinally would help to clarify the issue. Thus what has been discovered as a gene effect on brain structure or behaviour will shape hypotheses and subsequent studies on those age ranges.

The work in this thesis focused primarily on brain measures that could be derived from structural MRI scans, so it was not sensitive to sex hormone and genetic differences that are purely activational as might occur synaptically or neurochemically. Even where structural differences do occur, an association does not necessarily prove causality. For example, the gene might in addition to causing the observed structural differences also modify a third unmeasured factor that underlies disease risk or dimorphism in behaviour. While I acknowledge that such possibilities must be explored, I would argue that the gene to brain structure to behaviour link would be more fruitful in the presence of an association and given the importance of structural differences to lasting traits. A number of processes from

synaptogenesis and synaptic pruning to dendritic arborisation and myelination, all of which can modulate computation in some way, manifest with concomitant changes in structural properties such as volume and white matter integrity(Cline, 2001, Bailey and Kandel, 1993, Trachtenberg et al., 2002, Prayer et al., 2001). Furthermore structural changes are also likely occur secondary to experience-dependent plasticity induced by non-structural effects so could still be valid markers of the causal process. In many candidate gene studies investigating neural correlates of important behavioural genes, the regions implicated have been found to be important functionally in the relevant tasks and it would thus appear that much of structural change is significant and relevant.

The implication of sex hormone pathways in disease endophenotypes and behaviours is of particular therapeutic significance because of the broad range of pharmacological interventions available. There is a wide range of agonists and antagonists with varying specificities for the sex hormone receptors, and several hormone-based formulations are widely used in regimes for hormone replacement therapy and oral contraception. On the other hand, the implication of these pathways in personality and cognition will allow us to consider the subtle and perhaps ignored or as yet unconsidered influence such agents may have on the brains of individuals who take them for various other medical reasons. They could potentially pave the way for further studies on the range of psychiatric and cognitive symptoms that individuals with hormone disorders might experience. Eventually a broader understanding of this picture could lead to individualised regimes that improve symptoms while minimising pharmacological side effects.

6.3 Individual findings

6.3.1 PROGINS association with brain volume

So what insights can be garnered from the implication of the progesterone receptor in the genesis of sexual dimorphism in brain volume? In the first instance, the discovery of a

hormonal factor that specifically modulates brain volume lends further support to the statistical evidence countering the theory that larger male brain size is a purely allometric effect. A previous study found that variants of genes causing microcephaly, Abnormal SPindle-like microcephaly-associated (ASPM) and microcephalin, co-occur with haplogroup and thus ethnicity and likely account for some ethnic differences in brain size. Thus an important first step will be to determine whether frequency of genetic polymorphisms in the progesterone receptor pathway also varies between populations and whether these can account for haplogroup-based variation in sex differences in brain volume. It would also be important to replicate this finding in these populations. It would also be interesting to determine whether the progesterone receptor gene has undergone positive selection within humans and across species, and to see whether mutations in PGR, the gene for the progesterone receptor, might account for inter-species variation in brain size orthogonal to the allometric relationship.

The second factor in the second order PCA was correlated with brain volume and PROGINS genotype, accounted for about 6% of the individual variance in neuroticism and was negatively weighted for both aggressive and non-aggressive negative affect. I suggest that this is the component of neuroticism that accounted for the previously reported association between brain volume ratio and neuroticism (Knutson et al., 2001).

As previously mentioned, post-mortem studies have shown that sex differences develop *in utero* and up until the third year of life. So one would expect a genotype by age interaction to exist in sex differences in brain volume over this period. Thus this finding would be strongly corroborated if such samples were to be genotyped for PROGINS or if *in vivo* longitudinal studies of neonates and foetuses were to look for such an interaction with maternal progesterone, and foetal or neonatal genotype.

As the cascade of sexual differentiation is triggered by testicular differentiation or its absence, testosterone has been traditionally thought to be the main hormone in the organisation of brain sex differences. The absence of an influence of AR(CAG)n on a

fundamental measure of sex difference in brain volume highlights the breadth of the repertoire of factors and mechanisms by which the neuroendocrine axis induces sexual dimorphism. The preoptic area is frequently used as a model for the study of hormone-based sexual differentiation in rodents. Dramatically higher progesterone receptor immunoreactivity in the preoptic area has been observed in males as compared to females around birth. This jump in progesterone receptor expression was induced by prenatal exposure to testosterone propionate or synthetic oestrogen diethylstilbestrol, but not dihydrotestosterone propionate, which acts selectively on the androgen receptor. Additionally it is reduced in aromatase inhibition suggesting that this sex difference in progesterone receptor expression is caused by aromatised oestrogen (Quadros et al., 2002). Additionally, as has been mentioned earlier, the progesterone receptor has been shown to be important in the regulation of neurogenesis and neuroproliferation. The mechanisms, by which the mutation could exert opposing effects on those processes remain unclear although the promoter does include oestrogen responsive elements in addition and the mutation might exert pleiotropic effects which are of differing impact between sexes.

As mentioned previously, progestins are often given to prevent early spontaneous abortions and they appear, despite potential objections and confounds, to relate to subsequent academic achievement and motor development (Wagner, 2006). If a mechanism can be determined by which the progesterone receptor modulates brain development, then such regimes could be tailored to improve development. The ethics of non-disease related prenatal pharmacological intervention is an as yet unexplored territory which, though interesting to consider, is beyond the scope of this thesis.

6.3.2 Temporal lobe associations of AR(CAG)n and ESR2(CA)n

Investigating two previously reported gene associations with Alzheimer's disease, I found that ESR2(CA)n was negatively associated with bilateral hippocampal volume in females and showed a sex by genotype interaction in the lateral inferior temporal gyrus. AR(CAG)n showed a sex by genotype interaction after small volume correction from a

coordinate of sex difference, an association in females and across sex with hippocampal volume, and an age by genotype interaction with shorter alleles associated with faster age-related atrophy. I describe an association of ESR2 polymorphism (encoding oestrogen receptor beta) with hippocampal volume in females that appears to concur with the pattern of genetic risk ESR2 polymorphisms confer in Alzheimer's disease. This warrants further investigation into possible mechanisms and if this link truly is relevant to Alzheimer's disease pathogenesis. The CA repeat in the promoter region of ESR2 can be expected to have an effect on receptor expression and this should be confirmed in hippocampal tissue and by molecular genetic characterisation. On a systems level previous studies have shown it has an effect on serum oestrogen that might mediate this effect (Sowers et al., 2006, Scariano et al., 2004), but does it have the same effect on hormone levels within the brain? More crucially, activation of oestrogen receptor beta by oestrogen has been shown to be the main mediator for the effects of oestrogen on the hippocampal synaptic plasticity and memory using a knockout mouse model and using specific oestrogen receptor beta agonists (Liu et al., 2008). Further behavioural and functional studies could be done with this polymorphism and specific agonists to demonstrate that this effect explains increased volume in my cohort, while a link could be investigated between this important influence on normal function and possible neuroprotective mechanisms.

It can also be useful to consider the clinical implications of such an approach. Hormone replacement therapy has been extensively studied in clinical trials with Alzheimer's disease. While there is some degree of variation, the general consensus from larger studies has been that oestrogen-containing therapies in early as opposed to late post-menopause reduce the risk of Alzheimer's disease (LeBlanc et al., 2001, Resnick and Henderson, 2002, Henderson et al., 2005). This is somewhat consistent with an organisational or disease-modifying influence of oestrogen over the course of adulthood as my study might suggest, however this must be answered in a more conclusive way. An issue with hormone therapies is that sex hormones have multiple effects on several organs across the body, which often differ with stage of life (Brinton, 2005). Thus raised oestrogen levels could both be neuroprotective while raising risk for breast cancer. The gene approach to identifying involvement of specific receptors on a systems level could generate hypotheses for trials with much more specific molecular targets and discrete phenotypes as identified. "

6.3.3 Amygdala covariance

The amygdaloid nuclei are known to be sexually dimorphic, and the finding that amygdala volume covariance with that of the inferior temporal cortex and its laterality is sexually dimorphic was replicated here (Mechelli et al., 2005). Additionally, I find that negative covariance with the right inferior temporal gyrus in males is negatively correlated with AR(CAG)n, while amygdala covariance of the left inferior temporal gyrus volume in females is positively correlated with AR(CAG)n. These findings suggest that the androgen receptor may be involved in the development of these asymmetries and differential regional volume covariance between males and females. Such a mechanism would be further supported by the discovery that AR(CAG)n also influences white matter volume in the temporal stem, uncinate tract and fornix. Functionally, the amygdala shows sex-related hemispheric lateralisation in emotional processing with women activating the left amygdala more strongly and men activating the right amygdala more strongly when remembering emotional material or when viewing happy faces. A similar finding has been made with resting state PET (Kilpatrick et al., 2006). The reduced left amygdala responsiveness of women with Turner syndrome, lacking the X-chromosome (Skuse et al., 2005) could support this hypothesis, as the X-linked AR gene would then have reduced dose compared to women with two copies.

The amygdala covaries in a manner modulated by ESR1(TA)n with the volume of left deep nuclei and anterior tonsil of the cerebellum. This region is also larger in GM and WM volume and shows increased FA in females. The cerebellar fastigial nucleus has been shown to project to the amygdala and other temporal sites (Heath and Harper, 1974), and also the same hypothalamic neurons are connected to both the amygdala and the cerebellum (Dietrichs and Haines, 1986). Thus the cerebellum has known connections to limbic circuitry that might be shaped by oestrogen via action on long range projection neurons to and from the cerebellar nuclei. This proposal is of relevance because synaptic plasticity in the cerebellum is a necessary process in fear conditioning and extinction and it would be interesting to see whether oestrogen can modulate this process (Sacchetti et al., 2005).

6.3.4 Regional volumetric associations with sex hormone-related gene polymorphisms

I have discovered that temporal, somatosensory, medial and rostral prefrontal, subcortical, and cerebellar brain regions are sexually dimorphic and that the differential morphology is significantly associated with sex hormone-related genes. While the detailed regional comparisons and consideration of mechanisms are discussed in chapters three and four, I attempt to place the results in the context of other studies investigating the hormonal contributions to brain structure. The stria terminalis is the major output channel for the amygdala to the ventral medial nucleus of the hypothalamus and thalamus. The stria terminalis has been shown to be larger in heterosexual men than women. However this relationship is reversed in transsexuals such that transsexual male-to-females have female-normative volumes, while transsexual female-to-males have volume similar to those of heterosexual males (Zhou et al., 1995). This was shown to be due to the number of somatostatin-expressing neurons from the stria terminalis itself, as opposed to the innervations of the amygdala (Kruijver et al., 2000). This finding was explained in terms of loss of androgens as all of the male-to-female transsexuals had been orchidectomised and five were taking the antiandrogen cyproterone acetate. In concordance with this idea, the hypothalamus showed a positive association of AR(CAG)n in GM and FA, and also a negative association in females with ESR2(CA)n in GM, but as might be expected there was no association with the amygdala.

In a study of menstrual cycle neural plasticity, activity in the medial superior frontal gyrus correlated with both progesterone and oestrogen serum levels, while superior temporal gyrus activity was correlated with progesterone levels (Fernandez et al., 2003). Although no relative regional differences were observed with PROGINS, the superior temporal cortex was associated with AR(CAG)n, ESR1(TA)n and ESR2(CA)n, while the medial frontal lobe was associated with AR(CAG)n, ESR2(CA)n and CYP19(TTTA)n.

In a study of pubertal adolescents, oestradiol levels have been associated with grey matter decreases in the bilateral superior and left orbitofrontal gyri as well as the right inferior frontal and angular gyri and grey matter increases in right middle frontal gyrus and right inferior temporal gyrus in girls. There were no associations in boys with either oestradiol or androgen serum levels (Peper et al., 2009). The medial frontal clusters positively associated with ESR1(TA)n appear to overlap with the bilateral superior frontal gyri that decrease in volume with increasing serum oestradiol levels. ESR1(TA)n and ESR2(CA)n are negatively and AR(CAG)n positively associated with the inferior temporal gyrus, which was found to increase in volume with increasing oestradiol levels. Both this study in adolescents and another study in young adults found an association between oestradiol and the inferior frontal gyrus (Witte et al., 2009), although I found no such association in my study with hormone receptor polymorphisms.

In post-menopausal women, those who had never been on oestrogen therapy have smaller grey matter volume in the orbitofrontal cortices, cerebellum, right inferior frontal and precentral cortices and left paracentral cortex compared to young women and those on oestrogen therapy (Robertson et al., 2009). Except for the inferior frontal gyrus, these are regions similarly associated in my study with AR(CAG)n, ESR2(CA)n and CYP19(TTTA)n.

Thus except for the right inferior frontal gyrus, most regional volumes found to correlate with serum hormone levels are also associated with sex hormone-related gene polymorphisms. Of the regions I implicate, only the somatosensory and subcortical area volumes do not appear to be correlated with serum hormone levels.

6.3.5 CNTNAP2

The neural correlates of the risk allele for CNTNAP2 were characterised by VBM and voxel-wise comparisons of FA. As this is the first study to investigate brain correlates of

the gene, the *a priori* hypothesis was relatively broad. While brain effects follow the disease association in that significant effects were only apparent in homozygotes for the risk allele, they were not male-specific. The association with rostral prefrontal, occipital and cerebellar regions is in line with commonly affected brain regions in autism spectrum disorder and the rostral prefrontal and cerebellum were also influenced by sex and hormone-associated gene polymorphisms. As autism is a disorder of development, the timing at which CNTNAP2 influences these brain structures is of particular importance and may provide clues about why certain developmental milestones or behaviours are delayed or not acquired. The gene was also associated with mistrust and anger responsiveness in risk homozygotes, which can be considered to be a social measure of anger. Tractography using implicated brain regions as seeds will allow confirmation of the overall circuit implied anatomically by my results and could answer further questions about the difference in connectivity profiles between genotypes.

6.3.6 Gender role orientation

Gender role orientation as measured by masculinity over femininity was negatively associated with AR(CAG)n, positively associated with MAOA VNTR low expressing alleles and showed a sex interaction with 2D:4D. Both genes as well as their ligands or substrates have been linked to aspects of aggression and social dominance, which are considered in this framework to be masculine traits (Simon and Whalen, 1986, Simon and Lu, 2006, Buckholtz and Meyer-Lindenberg, 2008, Frazzetto et al., 2007, Connell, 2001). The presence of a candidate gene association with gender role orientation is particularly significant in light of its traditional presentation as a construct of socialisation. Previous studies have shown associations with masculinity and femininity with 2D:4D (Lippa, 2006, Csathó et al., 2003) and salivary testosterone levels (Baucom et al., 1985) and my study adds further evidence for a biological influence.

6.3.7 Neuroticism

The reasons for the choice of neuroticism in which to investigate sexual dimorphism in personality were twofold. Firstly of the big five factors, women score higher in neuroticism more consistently and strongly than other major facets of personality. Secondly, neuroticism is closely linked to risk of mood disorders such as depression and anxiety, which also have a higher incidence in women (Francis, 1993, Jorm, 1987, Fanous et al., 2002, Saklofske et al., 1995, Jardine et al., 1984). In identifying six components of neuroticism across questionnaires, I demonstrate that there is substantial variability in measures of neuroticism and suggest that finer analysis and characterisation of the constructs of personality are necessary to most effectively identify molecular and neural contributions. This is supported by the results of most recent genome-wide association studies that have found small effect sizes accounting for less than 1% of variance in personality, with very few associations surviving replication.

CYP19(TTTA)n is associated with the sexually dimorphic factor 1, comprising both aggressive and non-aggressive components of negative affect. The balance between oestrogens and androgens is influenced by the aromatase enzyme. Indeed endogenous levels as well as exogenous administration of androgens and oestrogens have been shown to substantially influence mood in a wide range of studies (Joffe and Cohen, 1998, Barrett-Connor et al., 1999, Anderson et al., 1992, Seidman et al., 2001, Miller et al., 2002). This influence is thought in part to involve serotonergic interactions (Amin et al., 2005, Joffe and Cohen, 1998). Thus it is not so surprising that the metabolising enzyme for these hormones should modulate a major trait controlling negative affect. This has significant clinical implications if certain parameters can be determined. Firstly, if sex difference is known to account for 10-20% of variance in neuroticism or aggression-related traits (Plomin and Foch, 1981), how much variance in mood and severity of depressive, anxiety or aggression-related symptoms across a range of disorders of mood and aggression can be accounted for by sex hormones? Secondly, what constitutes an ideal balance of sex hormones and how can this be achieved by pharmacological manipulation of aromatase and sex hormone receptor signalling?

A number of regions, many limbic and emotion-related, were associated with each factor. Other studies have found an association between neuroticism and grey matter volume and cortical thickness in the subgenual anterior cingulate cortex, which was associated with factors 1 and 2 in my first order analysis and factor 2 in the second order analysis (Blankstein et al., 2009). The hippocampus, associated with factor 2 of the second order analysis has also been commonly associated with neuroticism, with harm avoidance shown to associate with smaller right hippocampal volume (Yamasue et al., 2007). Another study found that stress in depressive patients caused white matter alterations in the hippocampus and individuals homozygous for the S allele of 5-HTTLPR with childhood neglect had smaller hippocampi (Frodl et al., 2010). Somewhat surprisingly I find no associations between hippocampal volume and any of the first order analysis factors. Harm avoidance has also been associated with larger left amygdala volume in females, but not in males (Iidaka et al., 2006), however the amygdala was not associated with any of the factors except factor 4, where a right ventromedial temporal lobe cluster appeared to include the amygdala. The right lateral inferior frontal gyrus and rostral prefrontal cortex bilaterally were associated with factor 1 of the first order analysis, while medial and rostral prefrontal cortices were associated with factor 2 of the second order analysis. Smaller left anterior prefrontal cortex has been found to correlate with harm avoidance in females (Yamasue et al., 2007), while the left prefrontal cortex has been shown to be smaller in depressive patients, but larger in healthy L allele carriers of 5-HTTLPR who experience childhood stress(Frodl et al., 2010). Many of the remaining regions have been shown to be important in emotional regulation and cognitive control of emotion(Ochsner and Gross, 2005, Ochsner and Gross, 2008).

6.3.8 Empathy

In analysing the psychometric relationship between gender orientation and neuroticism with empathy and other social traits, I found close links between the three sets of traits. In line with other studies where individuals scoring higher on femininity were more empathic and

expressed greater empathic concern, M/F or greater masculinity with less femininity was associated with decreased social affiliativeness and its subscales of emotional empathy and empathic guilt (Karniol et al., 1998, Watson et al., 1994).

Use of PCA to dissect statistically orthogonal components of neuroticism was particularly useful in clarifying the relationship between neuroticism and empathy, which has been equivocal on some counts (Del Barrio et al., 2004, Richendoller and Weaver, 1994, Guarino et al., 2007, Hekmat et al., 1974). I draw connections between different aspects of neuroticism and the affective and expressive components of empathy in this study. I would suggest a trait-based model of empathy involving three components. The first component comprises the underlying capacity to appreciate or represent negative affect relating to factor 1 of neuroticism. The second can be conceptualised as a lack of affiliativeness that is associated with increased preference for aggressive behaviour as opposed to non-aggressive expression of negative affect and this relates to factor 3 and M/F. The third can be understood as internal emotional absorption where the tendency to cope poorly and experience intensely one's own negative emotions causes impairment of emotional perception and less shared affect. This tendency might be to bottle-up negative emotions with factor 4 or blow up on the outside with factor 6 without being able manage them well internally.

Increasing social affiliativeness was associated with increased right lateral inferior frontal gyrus volume in men and with medial orbitofrontal and lateral temporal volume in women. This warrants further functional investigation as to whether these regions are disparately recruited in the expression of this trait or if dysfunction of those regions could lead to diminished social affiliativeness. ESR1(TA)n was negatively correlated with both lateral temporal cortex volume and social affiliativeness and it would be interesting to investigate in an ESR1 KO model as well as temporal lobe dialysis with estrogen agonists and antagonists whether changes to the oestrogen receptor alpha acts through temporal cortex and if that changes behaviours. An interspecies comparison of oestrogen receptor alpha immunoreactivity showed that reductions in oestrogen receptor alpha were associated with increased prosocial and affiliative behaviour in male rodents, which would be congruent

with the genotypic finding (Cushing and Wynne-Edwards, 2006). Another study of female knockout mice for oestrogen receptor alpha found that they had reduced ability to perform social discrimination and so modulate their social behaviour from affiliative to aggressive according to individual (Choleris et al., 2006). A similar temporal lobe region appeared to be activated when imagining oneself in embarrassing situations although not in situations where one did something morally wrong and it might be involved in negative social evaluation (Takahashi et al., 2004). Perhaps individuals who are better at imagining the affective consequences of social censure express greater empathic guilt and empathy. The identification of biological factors underlying social traits could conceivably pave the way towards treatment of social conduct disorders and psychopathy, where individuals lack a sense of guilt or conscience. These disorders have been previously linked to MAOA through numerous studies (Buckholtz and Meyer-Lindenberg, 2008) suggesting that normal variation in social affiliativeness could be a valid endophenotype.

6.4 Conclusions

In discovering genetic associations with sexually dimorphic traits in overall brain volume, regional GM and WM volume and FA, structural covariance, gender role orientation, neuroticism and empathy, I have identified possible factors and hormone pathways that are likely to be important for producing these sex differences. I also suggest potential mechanisms based on the interactions of these polymorphisms with other factors such as age and sex, as well as findings in the literature. With overall brain volume, I have found that PROGINS, a mutation that diminishes progesterone receptor function and expression, causes reduced brain volume in men and increased volume in women. I suggest that this is due to a differential effect on neuroproliferation and neurogenesis due either to pleiotropic effects linked to an oestrogen effect on transcription or to a differing hormonal milieu as androgens have been found to cause induction of progesterone receptor expression.

With temporal lobe volume and regional volumes in general larger GM and WM volume and sometimes FA are positively associated with AR(CAG)n, negatively associated with

ESR2(CA)n in females, and show a sex interaction with CYP19(TTTA)n. With AR, I suggest that a trophic influence appears to be related to expression of the unbound receptor as higher expression is associated with faster age-related atrophy and smaller volume, but other studies show that this is ameliorated in men with higher serum androgen. With ESR2, I suggest that there is a direct trophic influence of the bound receptor with oestrogen such that in females who have high circulating oestrogen, higher expression leads to larger volume. With CYP19, I suggest that the trophic influence depends on the prevailing levels of hormones originally synthesised such that there is an optimal hormonal ratio for a given starting equilibrium. I provide a hypothesis for a more elaborate mechanism by which the ratio of bound and unbound androgen and oestrogen receptors varies according to the available pool of hormonal ligands as determined by aromatase activity.

As hypothesised, I have discovered an association between gender role orientation and AR and MAOA genes shown in other studies to influence aggression. By breaking down neuroticism into components, I was able to identify an association with CYP19(TTTA)n in the sexually dimorphic factor 1, and to suggest that it exerts its influence through modulation of brain area BA10. This idea implies that the balance of oestrogens and androgens is important in influencing overall mood and the expression or mode of negative affect. By analysing the sex by 5-HTTLPR genotype interaction with various components of neuroticism, I attempt to resolve the equivocal association of serotonin transporter genotype and sex with neuroticism.

I further describe the relationships among gender role, neuroticism and social traits suggesting an overall model for empathy and social affiliativeness. I discovered that ESR1(TA)n is negatively correlated with social affiliativeness and carrying the low expressing allele of the MAOA VNTR is negatively correlated with emotional empathy. In addition to finding that disparate circuits appear to underlie trait affiliativeness in men and women, I identify the lateral temporal cortex as a likely region on which oestrogen receptor alpha may act to modulate social affiliativeness.

Thus I find associations that indicate a range of mechanisms in the development of sexually dimorphic traits. At the same time, I have investigated disease-relevant traits or endophenotypes that are sexually dimorphic. Using these traits as proxies for disease, I have identified parallel associations and their concomitant neural or genetic correlates. I first found that hippocampal volume and age-related temporal lobe atrophy, when used as surrogate markers for Alzheimer's disease risk, largely parallel previously shown gene associations with ESR2 and AR, albeit with a different sex interaction with AR. I then investigate the neural correlates of an autism-associated polymorphism of CNTNAP2 that confers risk in a male-specific manner on the premise that autism is a disorder involving abnormal development of brain structure. Finally, I have used neuroticism as a personality-based risk factor for mood disorders in order to identify genetic and neural correlates that might modify mood and risk for anxiety, depression or pathological aggression.

Eventually hypotheses and mechanisms proposed in this thesis will need to be tested, whether in direct replications, patient groups, functional and molecular studies or interventional and longitudinal studies. The potential for follow-up is wide, and from a clinical perspective I would argue that the pharmacological and imaging tools available to investigate these hypotheses in a therapeutic context are adequately precise to have direct translational implications.

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